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BIOCHEMICAL CHANGES DURING THE BREAKING OF DORMANCY IN LOBLOLLY PINE SEEDS, *PINUS TAEDA L.*

BY

WENDY L. SCHNEIDER

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

IN

PLANT MOLECULAR BIOLOGY

DEPARTMENT OF BOTANY

Edmonton, Alberta

FALL, 1993

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W. L. Schneider
Wendy L. Schneider
1033 55 Street
Edmonton, Alberta
T6L 1Y8

Date: August 30, 1993

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled BIOCHEMICAL CHANGES DURING THE BREAKING OF DORMANCY IN LOBLOLLY PINE SEEDS, *PINUS TAEDA L.*, submitted by Wendy L. Schneider in partial fulfillment of the requirements of the degree of MASTER OF SCIENCE in PLANT MOLECULAR BIOLOGY.

David J. Gifford

David D. Cass

Allen G. Good

Edwin A. Cossins

Janusz Zwiazek

June 24, 1993

I would like to dedicate this thesis to my family. My husband Brian along with our children, David, Jennifer, Timothy and Nicole. They were always very understanding when I was showing signs of stress. My extended family, my mom and inlaws, sisters, brothers, etc., also gave me encouragement and support. The effort that goes into hard work is always rewarded when the ones you care for show how proud they are of you. Thank you to my family.

ABSTRACT

Biochemical changes in loblolly pine seeds (*Pinus taeda* L.) were monitored throughout a chilling treatment (stratification) of 2°C. Embryos from the mature seeds used in this study were nondormant; however, they failed to germinate *in situ*. At 30°C these embryos showed the same ability to break down their stored protein reserves as their fully stratified counterparts when incubated on water agar. In addition, both embryo types showed similar patterns of protein synthesis during incubation on water agar. The seed coat played a major physical role in the maintenance of embryo dormancy in the unstratified seed. During a 42 day chilling period, seed germination rose from 19% to to a maximum of 78%; the greatest rate of increase occurred between 0 and 14 days . Moisture content per gram of fresh weight rose from 6% to 23% over the 42 day period. Lipids remained unchanged, as did the activity of the glyoxylate cycle enzyme, malate synthase. No significant changes in the total nucleic acids, DNA or RNA content of embryos or megagametophytes were observed. Extant proteins, both buffer-soluble and buffer-insoluble, did not change. Pulse-label studies of embryos and megagametophytes showed that the pattern of protein synthesis in embryos was similar throughout chilling treatment. The synthesis of some protein sets with molecular masses of 15 and 40 kilodaltons decreased following 14 days of chilling treatment. In the megagametophyte, equivalent proteins with molecular masses between 15 to 40 kilodaltons decreased in synthesis; however, there was a change in the synthesis of new

proteins between 14 and 21 days of chilling. One new set of proteins, in the 31 kilodalton mass region, increased synthesis until 28 days of chilling and then started to decrease between 35 and 42 days of chilling. Other newly synthesized proteins, with masses of 77.6, 67.6, 16.9 and 14.8 kilodaltons, followed similar patterns of synthesis. The synthesis of new proteins in the megagametophyte coincided with the loss of dormancy in the intact seed.

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ABBREVIATIONS LIST

ABA	abscisic acid
ATP	adenosine triphosphate
BSA	bovine serum albumin
DAI	days after imbibition at 30°C
DOC	days of chilling at 2°C
DTNB	dithionitrobenzoic acid
EC	Enzyme Commission
GA	gibberellic acid
IEF	isoelectric focusing
kDa	kiloDaltons
Lea	late embryogenesis abundant
ME	β-mercaptoethanol
OD	optical density
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error
TCA	trichloroacetic acid

I. INTRODUCTION

1.1 Loblolly Pine as a Resource

Loblolly pine (*Pinus taeda* L.) has become an important tree species in the south-eastern United States. As of 1982 loblolly pine surpassed slash pine (*Pinus elliottii*) as the most economically important species in managed forests. Loblolly pine at that time comprised more than 60% of the current planting in the southeastern United States (Zobel, 1982). Today, 80% of all the pines planted in the southern pine region are loblolly (Skinner, 1992). Because of its fast response to any kind of manipulative technique, loblolly is often the species of choice in studies aimed at improving wood quality and production. This response is found in loblolly in the laboratory setting as well as in the managed forest. Before forest management techniques improved loblolly stands, it had a poor reputation as a wood crop. In relation to this, Zobel (1982) emphasized the importance of understanding the seed biology of any crop plant. He stated how loblolly pine was thought to be one of the most genetically variable of all trees studied to that date. This variability was one of the most valuable attributes of loblolly but it also caused many difficulties in starting tree plantations. Zobel indicated that he thought that there were two main reasons:

1. Natural selection over a wide range of environments and over extended periods of time caused a

selection of the best adapted combinations. For example loblolly pine from the central Florida provenance is susceptible to cold while that from Maryland is quite cold-hardy. Thus, a seed from a central Florida provenance would grow poorly, or most likely not survive at all, in Maryland. These differences have their basis in the genetics of the plants.

2. Loblolly hybridizes easily with most other pine species that are in close ass ociation with it. Some genetically-based characteristics are transferred into loblolly pine, thus broadening its genetic base. Genetically the trees become more diverse through introgression (repeated backcrossing of hybrids to loblolly pine) but still retain their taxonomic identity.

The importance of understanding the seed source was not understood by foresters. Oftentimes loblolly seed was planted with no concern for source of seed. Consequently, many plantings of loblolly pine were categorized as poor or failures. After further research into why loblolly was such an outstanding crop in one area and not another, foresters then began to match seed source climate for climate. This resulted in increased productivity which has put loblolly in the forefront as a valuable resouce in forestry and in research.

With the development of new technologies, loblolly pine has changed from a species used for few restricted products, to one contributing heavily to nearly all kinds of fiber and solid-wood products (Zobel, 1982). Loblolly pine is highly desired for pulp and paper, plywood and whole wood (Kellison and Gingrich, 1982). It is also the major tree species used in pine research today. For example, Sederoff and his group at North Carolina State University are looking at the lignin biosynthesis pathway to further improve loblolly pine as a wood crop. Lignin, which is a constituent of the cell walls in woody species, is considered an undesirable component that is removed during chemical pulping. The extent of treatment required to remove lignin has a signficant effect on yield. Higher lignin content requires longer hydrolysis and bleach treatments which lead to higher costs and the environmental concern of bleach disposal. Since the chemical and physical composition of wood is genetically determined, these properties would be amenable to genetic engineering (Whetten and Sederoff, 1991). In relation to this, genes in the lignin synthesis pathway have been identified that code for cinnamyl alcohol dehydrogenase (O'Malley et al., 1989) as well as a second enzyme, phenylalanine ammonia lyase (Whetten and Sederoff, 1992) which is involved in the regulation of the pathway.

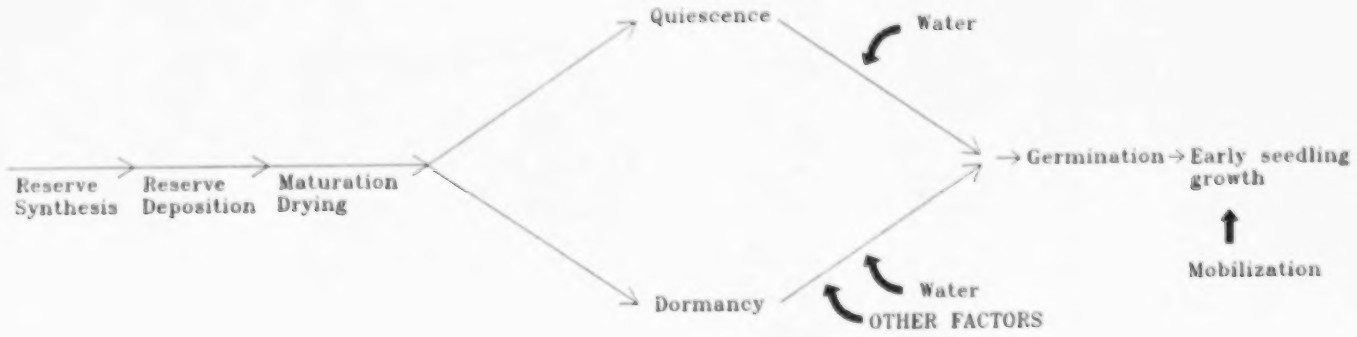
1.2 Barriers to Germination

A major concern in tree seedling nurseries is that many seed types require some kind of pretreatment before germination will

occur. Seeds that require a pretreatment such as chilling to promote germination are said to be dormant. Dormancy in seeds is a protective mechanism ensuring that germination occurs only when conditions are optimal. In this way, dormancy can be considered an important stage in seed development. During development, seeds follow a pathway toward germination that involves many steps (Fig. 1). In angiosperms, the pathway begins after fertilization with reserve synthesis, deposition of reserves and maturation drying; these stages are common to all angiosperm seeds (Bewley and Black, 1985). Gymnosperms are different, however. The initiation of reserve synthesis in relation to fertilization has yet to be determined. In conifers, reserve synthesis takes place in the megagametophyte, a maternally derived tissue that forms the nutrient source that will support the developing seedling (Foster and Gifford, 1974). In loblolly pine and other pines, lipids and proteins form the bulk of this reserve (Groome et al., 1991; Gifford, 1988). Once mature, the seed, depending on the type, will either enter a quiescent or a dormant period. If the seed is quiescent it usually requires only water to begin germination. After radicle emergence it then goes on to hydrolyze its reserves. If the seed is dormant, however, it will require water and 'other factors'. These other factors may include osmotic shock, acid or alkali treatment, plant growth substances, temperature change, scarification, chilling or changes in light intensity or wavelength (Bewley and Black, 1985).

While various hypotheses have been proposed to

Figure 1 Flow chart illustrating the general stages of seed development.



STAGES OF SEED DEVELOPMENT

account for the action of these pretreatments (Cohn, 1987) there is little understanding of how they work at the cellular or molecular level. Seed dormancy is influenced by the parental and the zygotic genotype. The genotype interacts with different environmental and physiological factors that occur from anthesis to dispersal, thus, inducing the kind of dormancy that the seed will exhibit (Kelly et al., 1992). Both dormant and nondormant seeds have the ability to imbibe water and perform *de novo* protein synthesis during the first few hours of imbibition, yet some biochemical and molecular restrictions prevent cell expansion and germination in dormant seeds (Goldmark et al., 1992). Kelly et al. (1992), state that there is a site of permeability on the testa that permits the breaking of dormancy in the natural environment. They suggest that this site, determined by ultrastructure studies during development, could be exploited in the nursery situation to break dormancy more naturally in economically-important plants such as crops. However, because of our lack of understanding of seed dormancy, current dormancy breaking mechanisms are generally empirical and consequently their applications are limited.

Some seed species require moist chilling treatment to break dormancy. It can be presumed that chilling treatment in the natural environment is the over-wintering period, thus, favouring germination in the spring. This moist chilling treatment is often referred to in the literature as stratification. After stratification, the seed overcomes the barrier to germination. Germination continues until the radicle emerges from the seed coat. Following germination, the food reserves

of the seed are mobilized to support the growth and development of the young seedling. The utilization of these reserves marks the end of the dependent heterotrophic stage of seed development and the beginning of autotrophic seedling growth.

1.3 Dormancy Defined

There are two kinds of seed dormancy; innate and imposed. In the former, the embryo fails to germinate when isolated from the seed; in the latter the embryo does germinate when isolated from surrounding tissues. Removal of dormancy is characterized by a widening of the range of conditions in which germination becomes possible. The kind of dormancy shown by seeds, be it innate or imposed, has no sharply defined boundaries. Sugar maple (*Acer saccharum*), barley (*Hordeum spp.*), pear (*Pyrus communis*), and apple (*Pyrus malus*) display true embryonic dormancy whereas other plants of these same genera/species, sycamore (*Acer pseudoplatanus*), apple (*Pyrus malus*, some cvs), barley (*Hordeum vulgare.*, some cvs) show an imposed dormancy (Bewley and Black, 1985). Many seeds in the Pinaceae, such as white spruce (*Picea glauca*), lodgepole pine (*Pinus contorta*), jack pine (*Pinus banksiana*), are not dormant; they only require water to germinate. Seeds of other members of the Pinaceae are dormant, including sugar pine (*Pinus lambertiana*), Douglas fir (*Pseudotsuga mensesii*), western white pine (*Pinus monticola*), eastern white pine (*Pinus strobus*), ponderosa pine (*Pinus ponderosa*), Japanese black pine (*Pinus thunbergii*), and loblolly pine (*Pinus taeda*).

To lose seed dormancy, loblolly pine requires a 60 - 90 day or 30 - 50 day, depending on the genotype, moist chilling treatment at temperatures below 5°C (Barnett, 1976).

1.4 Causes of Dormancy

1.4.1 Plant Growth Regulators and Dormancy Breaking

Plant growth regulators especially ABA and GA have often been implicated in the maintenance and breaking of seed dormancy (Bewley and Black, 1985). Results of Paul et al. (1973) indicated that the unstratified seeds of loblolly pine and those stratified up to a period of 28 days contained very little or no growth promoter (GA-like substances), and a relatively high concentration of an inhibitor (ABA-like substance). Following 42 days of moist chilling treatment, the promoter concentration gradually increased while the inhibitor level fell almost to zero. A high level of promoter but no inhibitor was detected in germinating seeds. The activity of auxin-like compounds was absent in mature seeds but slowly increased up to a period of 28 days and remained at this level for the subsequent stratification period. Very little changes in the levels of growth regulators were noted in warm stratified seeds as compared to the unstratified controls. It was concluded that chilling treatment undoubtedly modifies the endogenous balance of growth regulators that favours seed germination; however, this may not be the only change that occurs during cold stratification.

Another instance where a plant growth regulator may act to maintain dormancy is in seed of the grass *Bromus secalinas*. Goldmark et al. (1992) found a gene that was up-regulated in expression and whose mRNA accumulates when dormant seeds are imbibed (at room temperature in the dark) and remain growth-arrested. This transcript (pBS128) was found to be embryo-specific and regulated by ABA. These authors suggest the transcript product has a possible role in the maintenance of dormancy as it appears in a wide variety of wild grass species that utilize seed dormancy as a survival mechanism.

1.4.2 Protein Synthesis and Dormancy Breaking

Previous studies have indicated that the regulation of protein synthesis may play a role in the transition from dormancy to non dormancy (Bewley and Black, 1982). Hance and Bevington (1992) investigated protein synthesis in dormant embryos of sugar maple which is a seed that has cotyledon-imposed embryo dormancy. Sugar maple has a moist chilling requirement to break dormancy. Seeds that were cold treated at 4°C germinated after 27 days, whereas seeds incubated at warmer temperature (15°C) did not. Cold treatment at 4°C increased the embryo's capacity for *de novo* protein synthesis as measured by *in vivo* incorporation of 35S-methionine into new proteins. The authors found that at 4°C protein synthesis in the embryonic axis increased in a linear fashion prior to germination. It also increased in the cotyledons up until day 20 and then declined.

By employing IEF/SDS-PAGE they found that the levels of specific proteins were altered during chilling, primarily in the cotyledons. They speculated that one or more of these particular proteins may be associated with the inhibition of growth of the embryonic axis imposed by the cotyledons. Noland and Murphy (1986) found patterns of extractable proteins remained similar at 5°C and 25°C treatments in both embryos and megagametophytes. As well, they noticed an increase in the capacity for protein synthesis in embryos of sugar pine during cold treatment at 5°C as measured by incorporation of 3H-leucine. The authors demonstrated that this increase in the capacity for protein synthesis generally paralleled an increase in germinability. However, amounts of individual proteins seemed to vary between 5°C and 25°C embryos. This suggests the possibility that temperature affects the rate of synthesis and/or degradation of individual proteins in sugar pine embryos. If this is the case, then understanding the physiological mechanism of dormancy in sugar pine seeds could be significantly advanced by characterizing the individual proteins involved in this process.

While many researchers (Marcus et al., 1966; Brooker et al., 1977; Satoh and Esashi, 1979; Dommes and van de Walle, 1983; Bewley and Black, 1985) have suggested that protein synthesis appears obligatory for seed germination in most, if not all species, it is not clear whether the regulation of protein synthesis is involved in seed dormancy. In several cases, equivalent or higher rates of protein synthesis have been reported in dormant seeds than in non dormant seeds (Chen and Varner, 1970; Efron et al., 1971; Fountain and

Bewley, 1973; Ching, 1975). In such instances, it has been hypothesized that dormancy may be controlled by blocking or unblocking synthesis of particular proteins involved in specific processes. As mentioned earlier, Goldmark et al. (1992) identified a transcript whose product was believed to play a role in the maintenance of dormancy. Also, there may be specific enzymes required to break dormancy. These enzymes, or gene products, would act to inhibit the synthesis, or hydrolysis, of proteins that prevent germination (Satoh and Esashi, 1979). Specific enzymes, whose activity may be critical to the dormancy breaking process, include peroxidase (Tao and Khan, 1976) and aminopeptidases (Noland and Murphy, 1986). Noland and Murphy (1986) argued that the *de novo* synthesis of a germination-inhibiting aminopeptidase may be involved in maintaining sugar pine seed dormancy. They found that 5°C moist-cold stratified embryos had a significantly higher aminopeptidase activity. They postulated that the higher activity may be an indication of the destruction or modification of dormancy-maintaining protein(s). The rate of degradation of two dormancy-maintaining proteins of *Agrostemma githago* embryos was considerably more rapid in nondormant, after-ripened embryos than in dormant embryos (de Klerk, 1982). This suggests the development of the ability to hydrolyze or modify specific proteins may be important in the dormancy breaking process.

1.4.3 Physical Constraint, Oxygen Availability and Dormancy Breaking

Dormancy as caused by physical constraint has been studied extensively in pine (Barnett, 1972; Barnett, 1976; Carpita et al., 1983; Dunlap and Barnett, 1983;). It was reported that although the seed coat acts as a physical barrier against megagametophyte swelling and subsequent radicle emergence, it alone does not make the seed dormant. There are other physiological and biochemical phenomena that may contribute to dormancy. The description of the mechanism of dormancy given by Chen and Thimann (1966) for *Phacelia tanacetifolia* attributed dormancy to a balance between mechanical constraint by the endosperm and expansive force of the embryo. Stratification may not just alter seed coat structure; metabolic changes are thought to take place in the embryo and megagametophyte, during stratification, that increase the growth potential of the embryo (Paul et al., 1973; Kozlowski, 1971). This increased growth capability may enable the loblolly embryo to rupture the coat and thus mark the end of germination.

Within a seed the embryo is surrounded by many layers of tissue that may impede the entry of oxygen; the escape of carbon dioxide may be prevented by these same tissues. It has been speculated that if increased oxygen concentration is required for germination then gas exchange may play a role in seed dormancy. This is not universally true, however. In charlock (*Sinapis arvensis*) and cocklebur (*Xanthium pennsylvanicum*) for example, it was found

that the embryo required a very low partial pressure of oxygen to germinate. The seedcoat was sufficiently permeable to oxygen to meet these needs, therefore, gas exchange was not a factor in determining the germinability of these seeds. In these cases, the imposition of dormancy by the seedcoats involved more than limiting oxygen supply (Porter and Wareing, 1974; Bewley and Black, 1985).

Bewley and Black suggested that if oxygen does play a key role in dormancy-breaking it does so not by increasing respiration, but rather as an oxidizer of inhibitors that may be present. This is supported by data from isolated embryo experiments with cocklebur (Porter and Wareing, 1974). There was no substantial increase in oxygen uptake, yet the embryo germinated. This implied that the inhibitor was leached out freely from the embryo and into the surrounding environs. The impermeability of coats to gaseous exchange may reduce the availability of oxygen to the embryo leading to an accumulation of intermediate products formed by fermentation (Thornton, 1945). Ching (1959) found that imbibition of water is coupled with linearly increasing respiratory activity. Seeds, such as lettuce (*Lactuca sativa*), rice (*Oryza sativa*), wild oats (*Avena spp.*) and barley, that were placed in a hyperoxic environment showed greater germinability, yet, did not show a difference in rate of formation of ATP, i.e. an increase in respiration (Simmonds and Simpson, 1971; Bewley and Black, 1985). Conversely, charlock will only form germination inhibitors in low oxygen concentrations.

Seedcoats limit oxygen uptake in several pines including eastern white pine (*Pinus strobus*) and Jeffrey pine (*Pinus jeffreyi*)

(Porter and Wareing, 1974). However, data that support the seedcoat impermeability hypothesis have also been interpreted as indicating that mechanical restriction is an important factor in seed dormancy. The increased speed of germination in stratified and clipped seeds may result from easier rupture of the coat by the swelling of the imbibing embryo (Hoff, 1987). Moisture uptake can then more rapidly continue to the level needed for germination. Respiration seems related to moisture uptake and, if the coats restrict this uptake, respiration levels remain low. This hypothesis is supported by the fact that less dormant longleaf (*Pinus palustris*) and slash pine seeds are not so closely restricted to an equilibrium moisture content, but continue to absorb moisture slowly (the rate seemingly related to the degree of dormancy) until germination occurs (Barnett 1969). In loblolly pine, respiration followed the pattern of water absorption in decoated seeds, i.e. respiration increased proportionately with increased water content. However, respiration was at a low level in stratified, clipped and unstratified seeds until germination began; there seemed to be no causal relationship between respiration and coat permeability (Barnett, 1972). Barnett concluded that dormancy is likely to be the result of mechanical constraint rather than by limited water or gas exchange. Therefore, it can be suggested that chilling treatment may aid the seed in overcoming some mechanical obstacle to germination. In summary, there are several possible constraints that conceivably act together to maintain the intact seed in a dormant state.

1.5 The Present Study

The pine seed is an ideal system for studying interrelationships such as the roles played by the embryo, megagametophyte and other tissues. Other researchers have commented on the close physical association between the embryo and the megagametophyte and how communication may exist between the two systems (Bewley and Black, 1985; Tabor and Barnett, 1987). Seed development is ideally suited for the study of gene expression for this is where dynamic changes are occurring to prepare the newly formed sporophyte for autotrophic existence. Some of the changes occurring are at the translational level, monitored as changes in extant proteins, and some are at the transcriptional level, monitored as changes in nucleic acids. In the present study, changes occurring during chilling treatment were monitored, for that is where the transformation from dormancy to non dormancy occurs in loblolly pine seeds.

1.5.1 The Intact Seed

The first step in studying the transition from dormancy to non dormancy was to specify the type of dormancy displayed in loblolly pine seeds. In the literature it is well documented that dormancy in pine is imposed (Larsen, 1925; Barnett, 1972; Barnett, 1976; Hoff, 1978; Carpita et al., 1983; Dunlap and Barnett, 1983; Pitel and Wang, 1985). To address where the transition from dormancy to non dormancy was occurring, I monitored changes through chilling treatment in the embryo and megagametophyte. Specifically, changes

in percent germination and moisture content were compared. This addressed whether or not germinability increased with time spent in moist chilling treatment. Also monitored during chilling treatment was lipid and protein content to see if these seed reserves were being broken down. To further substantiate if lipid breakdown was occurring, and whether or not the glyoxylate cycle was operating, malate synthase activity throughout chilling treatment was determined. Proteins were measured both qualitatively and quantitatively throughout chilling treatment to determine if breakdown was occurring. The synthesis of new proteins may be a requirement for dormancy breaking, therefore, embryos and megagametophytes were pulse-labeled with 35S-methionine throughout chilling treatment to see if *de novo* protein synthesis was occurring during the transition from dormancy to non dormancy. As a further display of dormancy, nucleic acid content should remain static, so to examine this, nucleic acid content was quantified during chilling treatment. It has been stated previously that loblolly pine seeds show imposed dormancy. To determine which tissues were imposing the dormancy, seed parts were removed sequentially and the seeds placed on wet filter paper to assess germination. It has been suggested (Asakawa, 1959) that an overabundance of water may hinder germination, so to test this hypothesis, seeds *sans* their integuments were placed on sterile, wet filter paper and on water agar and germinability was assessed.

1.5.2 Isolated embryo experiments

Embryos of loblolly pine seeds are nondormant. Mature seed embryos and fully stratified seed embryos were isolated and their germination and growth compared. The two embryo types were further compared by testing their physiological abilities. The strategy entailed looking at their respective abilities to degrade storage proteins as well as synthesizing *de novo* proteins as tested by the incorporation of ^{35}S-methionine while placed in appropriate conditions for germination.

II. METHODS AND MATERIALS

2.1 Chemicals

Radioisotopes such as ^{35}S-methionine and ^{14}C-methylated protein standards and BCS liquid scintillant were obtained from Amersham Canada Inc, Mississauga, Ontario. En^{3}Hance was supplied by Dupont Canada, Mississauga, Ontario. Trizma® base, Rifampicin and Amphotericin B were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Difco Laboratories, Detroit, Michigan, USA supplied bacto-agar. Protein molecular weight standards and ampholytes were supplied by BioRad Laboratories, Mississauga, Ontario. Silica gel desiccant 6-16 mesh was supplied by Fisher Scientific Company, Edmonton, Alberta. Other chemicals were supplied from companies such as Sigma Chemical Company, St. Louis, Missouri, USA; Fisher Scientific, Edmonton, Alberta; BDH Inc., Edmonton, Alberta; BioRad Laboratories, Mississauga, Ontario.

2.2 Equipment

The LS6000TA Scintillation counter, microfuge E®, J2-21 M/E Centrifuge and Du-60 Spectrophotometer were obtained from Beckman Instruments, Inc., Palo Alto, California, USA. The Eppendorf centrifuge 5415 was supplied by Brinkmann Instruments, Inc., Westbury, New York, USA. The incubators for seed germination

were acquired from Controlled Environments Ltd., in Winnipeg, Manitoba. Electrophoresis and gel drying equipment was supplied by BioRad Canada, Mississauga, Ontario. Filtration systems were obtained from Millipore Corp., Bedford, Massachusetts, USA. Kimpak® was supplied by Seedboro Equipment, Chicago, Illinois, USA. Plastic beakers (Nalgene®) were supplied by Nalge Company, a subsidiary of Sybron Corporation, Rochester, New York, USA.

2.3 Seed Acquisition and Sterilization

Half-sib loblolly pine seed (*Pinus taeda* L.) from a first generation select tree was a generous gift from Westvaco Corporation, Summerville SC, USA. The seed was collected from clone 11-9, which had been open pollinated, and collected in the fall of 1984. When the seeds were received, they were immediately placed and kept at -20°C, on the advice of Westvaco Corporation. When required, seeds were removed from -20°C, weighed and then sterilized following one of two different methods; surface-sterilized using bleach or a more vigorous approach using hydrogen peroxide (Molina and Palmer, 1982). In the case of bleach, the seeds were covered and swirled in 1% sodium hypochlorite for 1 minute, if less than 50 g, and 2 minutes if more than 50 g. When more vigorous sterilization was required, as in the case of excised embryo work, the seeds were weighed and placed in cheesecloth. They were then placed under running tap water for 30 minutes, swirled in a Tween 20 solution for 30 minutes and rinsed under running tap water for an additional 30 minutes. They were

then placed in enough 30% hydrogen peroxide to cover them and shaken for 1 h. At this time they were thoroughly rinsed with sterile, deionized water to remove all traces of hydrogen peroxide.

2.4 Percent Germination

To determine percent germination, seeds were surface-sterilized with sodium hypochlorite as mentioned above, and then placed in an autoclaved, sealed, plastic container (Sterilite®, 25 cm long X 17-1/2 cm wide X 4-1\2 cm deep) with moist Kimpak®. They were kept in the dark at 2±0.5°C and every 7 days, from day 0 through day 42, 100 seeds were taken from the cold treatment and placed in an autoclaved germination tray (Rubbermaid®, 13 cm diameter, 4 cm deep) with moist kimpak and placed at 30°C in a continually illuminated seed germinator (19 mmol $m^{-2}s^{-1}$). After 7 days at 30°C the seeds were counted as germinated if the radicle had emerged from the seed coat.

2.5 Moisture Content During Chilling Treatment

Water content determinations through chilling treatment were as described by D.G.W. Edwards (1987). A 50 mL Nalgene® beaker, (oven-safe) covered with a square of aluminum foil was weighed. Twenty seeds were evenly distributed over the bottom of the container. The container, cover and contents were immediately

reweighed. They were placed in a 103°C (± 2°C) oven with the cover beneath the container. The seeds were allow to dry for 17 hours (±1 hour). After removal from the oven the cover was placed upon the container and the device put in a covered desiccator (large beaker filled with silica gel desiccant) for 30 - 45 minutes . The container, cover and contents were, again, weighed and the following calculations were performed to determine moisture content as a percentage by weight:

$$\frac{(M2 - M3)}{(M2 - M1)} \quad X \quad 100$$

M1 is the weight (in grams) of the container with cover
M2 is the weight of the container, cover and seeds before drying
M3 is the weight of the container, cover and seeds after drying.

2.6 Nucleic Acid Extraction and Total Nucleic Acid Determination

The method of Durzan *et al.* (1971) was followed for the extraction procedure: 20 seed parts were ground in a mortar and pestle on ice using 0.5 mL of 95% ethanol. The slurry was transferred to a 1.5 mL microcentrifuge tube. Another 0.5 mL of 95% ethanol was added to the mortar and pestle as a rinse and added to the slurry. The mixture was spun at 14,000 rpm on an Eppendorf centrifuge in a 4°C cold room for 20 minutes. The pellet was

resuspended in 1 mL of ethanol:ether (3:1 v/v) and placed in a 70°C water bath for 3 minutes. It was then spun again for 10 minutes. This was repeated 3 times or until all fatty acids and pigments, where present, were removed. The resulting pellet was resuspended in 1 mL of 95% ethanol once and centrifuged as before. The pellet was then resuspended in 1 mL of 70% methanol and centrifuged as before. The resulting pellet was brought up in 1 mL of 2.5% perchloric acid on ice. The mixture was centrifuged for 15 minutes at 14,000 rpm at 4°C. The pellet was finally resuspended in 1 mL of 10% perchloric acid, and placed in a 70°C water bath for 30 minutes, centrifuged 15 minutes and the supernatant was used for the diphenylamine assay and to determine total nucleic acids. For total nucleic acid determination, the supernatant was first diluted 1:1 with deionized water and an OD at 260 nm was obtained.

A standard curve, at an absorbance of 260 nm, was plotted to determine the quantity of total nucleic acid by preparing selected concentrations of calf thymus DNA in 5% perchloric acid.

2.7 Diphenylamine Assay to Determine DNA Content

The Giles and Myers (1965) method, with modifications according to Gifford *et al.* (1991), was applied for quantifying DNA in loblolly pine throughout cold treatment. From the above nucleic acid extraction procedure, 0.2 mL of supernatant in 10% perchloric acid was added to 0.2 mL of deionized water plus 0.6 mL of 5% perchloric

acid. This resulted in a 1 mL solution of nucleic acids in 5% perchloric acid. The assay to determine DNA quantity calls for 2 mL of 4% diphenylamine (freshly prepared in glacial acetic acid) to be added to the 1.0 mL test supernatant in 5% perchloric acid followed by 0.1 mL of aqueous 1.6 mg/mL acetaldehyde. After incubation in a 30°C water bath overnight the OD at 595 and 700 nm was determined.

A standard curve was constructed using the same concentrations of calf thymus DNA as in the total nucleic acid standard curve. The DNA concentration was plotted against the difference in OD between reading at 595 nm and 700 nm. Using this curve, the difference in OD between 595 nm and 700 nm of the sample following the diphenylamine reaction was compared and the total DNA was determined. RNA was determined by subtracting the DNA content from the total nucleic acid fraction.

2.8 Malate Synthase Assay

2.8.1 Enzyme Extraction Procedure

Since the megagametophyte has the majority of the storage lipid reserves in loblolly pine, it was this tissue that was assayed for malate synthase (EC 4.1.3.2, L-Malate glyoxylate lyase, CoA-acetylating) activity through chilling treatment. Seeds were collected every seven days, 10 megagametophytes were removed and

ground in 1 mL of buffer containing 100 mM potassium phosphate and 6 mM magnesium chloride at pH 7.0 at 4°C. The slurry was transferred to a 1.5 mL microcentrifuge tube and spun at 14,000 rpm for 20 minutes in a 4°C cold room. The supernatant was carefully removed and used in the assays. This extraction procedure was also followed to obtain cell-free extracts of megagametophytes taken from seeds following imbibition at 30°C for malate synthase assays (see Results section).

2.8.2 Enzyme Assay

In oil seeds such as pine, the products of β-oxidation are metabolized by the glyoxylate cycle in an organelle called the glyoxysome. Malate synthase is a key enzyme of this cycle. The assay for malate synthase activity exploits the mechanics of this pathway by measuring the liberation of the sulfhydryl groups after the deacetylation of acetyl coenzyme A (acetyl coenzyme A-SH). CoenzymeA-SH in turn, reacts with DTNB causing a color change that is measurable on a spectrophotometer. All enzyme work was performed in triplicate using the bleach sterilized seeds. The malate synthase assay was from Hock and Beevers (1966), the pH optimum for loblolly pine was determined to be 7.7 and the concentration of substrate greater that 300 µg/mL. The assay called for 77 mM Trizma® Base (pH 7.7), 7.7 mM magnesium chloride, 1.5 mM DTNB, 180 mM acetyl coenzyme A, 100 µL supernatant containing enzyme, water and 20 mM sodium glyoxylate in a total volume of 1.3 mL. The spectrophotometer was blanked with water in place of the sodium

glyoxylate. The DTNB was solubilized in absolute ethanol; fresh pipet tips were used each time in adding DTNB to the assay volume as it precipitates very easily. The sodium glyoxylate was added last to the assay volume and the colour change was monitored spectrophotometrically as a change in absorbance at 412 nm. One Unit of malate synthase activity is the amount of enzyme which releases 1 nmol/min of free coenzyme A at room temperature (22°C); the extinction coefficient used was 13.6 X $10^3 M^{-1} cm^{-1}$ (Miernyk, et al., 1979).

2.9 Lipid Assay

Total lipids were extracted according to Becker *et al.* (1978) and Ching (1966) and as modified by Groome *et al.* (1991). One gram of megagametophytes were ground in 5 mL methanol:chloroform (2:1 v/v). The mixture was spun at 10,000 rpm (7,800g) in a Beckman J2-21M/E for 10 minutes at 20°C. The supernatant was decanted and the pellet re-extracted by grinding again in 5 mL methanol:chloroform. The supernatants were pooled in a separatory funnel and an equal volume of 2 M potassium chloride was added. This was shaken vigorously and the phases allowed to separate. The organic phase (bottom, lipids) was drained into preweighed beakers. The aqueous phase was washed 2 times with 3.5 mL of methanol:chloroform and the organic phase was collected each time in the beakers. The beakers were left overnight in the fumehood to evaporate solvents and then placed in a 50°C oven until a constant

weight was reached; approximately 30 h.

2.10 Electrophoresis

2.10.1 Protein Extraction

All protein extractions were carried out at 4°C as per Gifford (1988). Ten seed parts were ground in a small mortar with 0.5 mL extraction buffer containing 50mM sodium phosphate at pH 7.5 including 0.1 mM leupeptin as a protease inhibitor. The slurry was placed in a 1.5 mL microcentrifuge tube and centrifuged at 14,000 rpm in an Eppendorf microcentrifuge for 20 minutes at 4°C in a cold room. The lipid layer was carefully removed and the supernatant containing the buffer-soluble protein fraction removed. The pellet was washed 3 times with 0.5 mL of extraction buffer, each time centrifuging for 5 minutes at 14,000 rpm at 4°C. After the final washing, 0.25 mL of extraction buffer and 0.25 mL of Laemmli buffer (62.5 mM Tris-HCl {pH 6.8} containing 2% SDS {w/v} and 10% glycerol {v/v}; Laemmli 1970) was added to the pellet. The pellet was resuspended and heated at 95°C for 5 minutes in a block heater water bath. The mixture was centrifuged on a Beckman microcentrifuge at 14,000 rpm for 15 minutes at room temperature (22°C). The supernatant contained the buffer-insoluble protein fraction. Buffer-soluble and insoluble proteins were quantified using the Folin and Ciocalteu reagent (Lowry *et al.*, 1951) with BSA as the protein standard.

2.11 One-dimensional SDS-PAGE

2.11.1 Coomassie Blue Staining Procedure

Prior to electrophoresis, the soluble protein fraction was mixed with an equal volume of Laemmli buffer and heated at 95°C for 5 minutes on a block heater water bath. The insoluble protein fraction was used directly. Appropriate dilutions used Laemmli buffer with or without 5% ME (for non-reducing or reducing conditions, respectively). SDS-PAGE was carried out on 0.75 mm 12% polyacrylamide gels using a BioRad mini-electrophoresis system according to the manufacturer's specifications. The run was carried out at a constant 200 v. Gels were loaded with 5 µg of buffer-insoluble protein or 15 µg of buffer-soluble protein. Once the gel run was complete, gels were stained for 30 minutes in Coomassie blue R (Burk *et al.*, 1983), followed by two 40 minute destaining steps in 25% ethanol/8% acetic acid (v/v) and stored in 5% methanol/8% acetic acid (v/v) prior to drying. Once thoroughly destained, the gels were dried on Whatman 3mm chromatography paper at 80°C for 2 hours. If the gels were to be quantified by scanning densitometry they were dried between two pieces of clear acetate at 80°C for 2 hours. Scanning densitometry (Beckman DU-60 Spectrophotometer) was performed at 560 nm. Protein molecular weight standards were run on the gels as markers and included: phosphorylase b at 97.4 kDa; bovine serum albumin at 66.2 kDa; ovalbumin at 45.0 kDa; carbonic anhydrase at 31.0 kDa; soybean trypsin inhibitor at 21.5 kDa and lysozyme at 14.4 kDa (Weber and Osborne, 1969).

2.11.2 Fluorography Procedure

After the soluble proteins were extracted, 200 µL of the supernatant was removed and diluted with 200 µL Laemmli buffer containing 5% ME. This extract was then heated at 95°C for 5 minutes on a block heater water bath. Prior to SDS-PAGE samples were diluted down to the appropriate number of dpm using Laemmli buffer containing 5% ME. After SDS-PAGE, the gels were fixed in a 30% methanol/7% acetic acid (v/v) solution followed by soaking for two 60 minute periods in En^{3}Hance. The gels were then thoroughly rinsed with water and then gently shaken in water for a few minutes to ensure complete precipitation of the fluor chemicals. They were dried onto Whatman 3mm chromatography paper at 60°C for 2 hours and exposed to x-ray film (Kodak, X-Omat AR-5) for autoradiography at -70°C (Gifford and Bewley, 1984). The standards that were run with these gels were [14-C] methylated and included: myosin at 200 kDa; phosphorylase b at 92.5 kDa; bovine serum albumin at 69.0 kDa; ovalbumin at 46.0 kDa; carbonic anhydrase at 30.0 kDa and lysozyme at 14.3 kDa (Weber and Osborne, 1969).

2.12 Two-dimensional IEF/SDS-PAGE

For two dimensional IEF/SDS-PAGE, enough solid urea was added to 200 µL of the soluble protein fraction to make a 9.5M solution. This was dissolved under hot, running tap water. Prior to electrophoresis, samples were diluted down to 2 times the desired dpm using a solution containing 2% Triton X-100 (v/v), 5% ME (v/v) and 9.5M urea. This solution was further diluted 1:1 with a solution containing 9.5M urea, 2% Triton X-100, 5% ME, 1.6% Bio-Lyte 5/8 ampholyte and 0.4% Bio-Lyte 3/10 ampholyte. Tube gels were cast in 75 mm (length) by 1 mm (diameter) microtubes. The gel solution contained 9.2 M urea, 4% acrylamide, 1.6% Bio-Lyte 5/8 ampholyte and 0.4% Bio-Lyte 3/10 ampholyte. The samples were overlayed with a solution containing 4.5 M urea, 0.8% Bio-Lyte 5/8 ampholyte, 0.2% Bio-Lyte 3/10 ampholyte, and 0.0025% (w/v) as a final concentration of bromophenol blue. Gels were loaded by dpm. Two-dimensional gel electrophoresis was as per Bio-Rad Laboratories instructions for their mini tube gel system. For first dimension IEF, the upper chamber of the gel apparatus contained 20 mM sodium hydroxide; the lower chamber 10 mM phosphoric acid. After separation by IEF, proteins were separated in a second dimension using SDS-PAGE using previously described running conditions. Once gel runs were complete, gels were treated in the same manner as in SDS-PAGE; i.e., fixing, En3Hance, water rinse and dried onto Whatman 3mm chromatography paper for 2 hours at 60°C. Autoradiography was the same as for the SDS-PAGE gels.

2.13 *In vivo* Protein Labeling

Ten seed parts were labelled with 100 µCi (3 X 10^9 Bq) in a total volume of 100 µL of ^{35}S-methionine (Specific activity 37 TBq/mmol, 1000 Ci/mmol) in a sterile covered petri dish. The tissue was exposed to the label for 3 hours. The seed parts were then ground, on ice, in 0.5 mL of 50 mM sodium phosphate buffer (pH 7.5) containing 0.1 mM leupeptin. The slurry was placed in a 1.5 mL microcentrifuge tube and centrifuged in an Eppendorf centrifuge at 14,000 rpm for 20 minutes at 4°C. The lipid layer was carefully removed and the supernatant removed. The supernatant was treated in accordance with the type of characterization. It was either used directly for radioactivity incorporation measurements using TCA precipitation or was prepared for electrophoresis. For SDS-PAGE, an equal volume of Laemmli buffer containing 5% ME was added and the mixture heated for 5 minutes on a block heater water bath at 95°C. For IEF/SDS-PAGE analysis, the supernatant was made 9.5 M using solid urea.

Incorporation of radioactivity into protein was determined by TCA precipitation (Groome *et al.*, 1991). Ten µL of supernatant was spotted in the center of a Whatman GF/C glass filter that was pinned on foiled covered styrofoam. The filter was dried with forced air. Once dry, the filter was washed 6 times with 5 mL cold 10% TCA using a Millipore filtration system. All filters were then placed in a microfuge tube containing 1 mL of 10% cold TCA and boiled for 15 minutes in a block heater water bath to hydrolyze amino-acyl-tRNA

bonds. The filters were then washed, under vacuum, 4 times with 5 mL cold TCA followed by washing 4 times with 5 mL cold absolute ethanol. After drying as above, the filters were placed in 10 mL BCS scintillant and counted on a Beckman LS6000TA liquid scintillation counter.

2.14 Isolated embryo and sequential removal of tissue studies

Embryos were excised from mature unstratified seed and fully stratified seeds, then imbibed in Petri dishes containing 3% unsupplemented water agar in a continuously illuminated seed germinator (19 mmol $m^{-2}s^{-1}$) at 30°C for up to 4 days. The agar contained amphotericin B as an antifungal as well as rifampicin as an antibiotic in concentrations suggested by the manufacturer for plant culture.

The sequential removal of tissue experiment was performed by placing the following treatments on sterile, damp, Whatman #1 filter paper in Petri dishes placed at 30°C in an illuminated seed germinator (19 mmol $m^{-2}s^{-1}$).

(1) outer and middle layer of integument removed
(2) all three layers of integument removed
(3) all three layers of integument and nucellar cap removed
(4) hard seedcoat and nucellar cap removed
(5) whole seed served as a control.

In a second set of experiments 3% water agar containing rifampicin and amphotericin B was substituted for the filter paper. The following were sampled:

(1) isolated embryos

(2) all tissues external to the megagametophyte removed.

III. RESULTS

3.1 Percent germination

The mature loblolly pine seed used in this study showed 19±4% germination prior to chilling treatment, and 78±2% germination following this procedure (Fig. 2A). Through chilling treatment it was noted that with 0 to 14 days of cold treatment there was a sharper increase in percent germination, 19±4% to 54±5%, and thereafter germination increased more gradually.

3.2 Moisture content through chilling treatment

During chilling, the relative water content per gram of fresh weight of the whole seed rose from 6±0.5% to 23±0.2% (Fig. 2B) indicating that hydration is also required for germination. After 35 days of chilling the moisture content appeared to level off.

3.3 Lipid content and malate synthase activity through chilling treatment, and malate synthase activity following germination

The megagametophytic lipids remained unchanged through chilling treatment (Fig. 3). The lipid content was 728±57 mg per gram

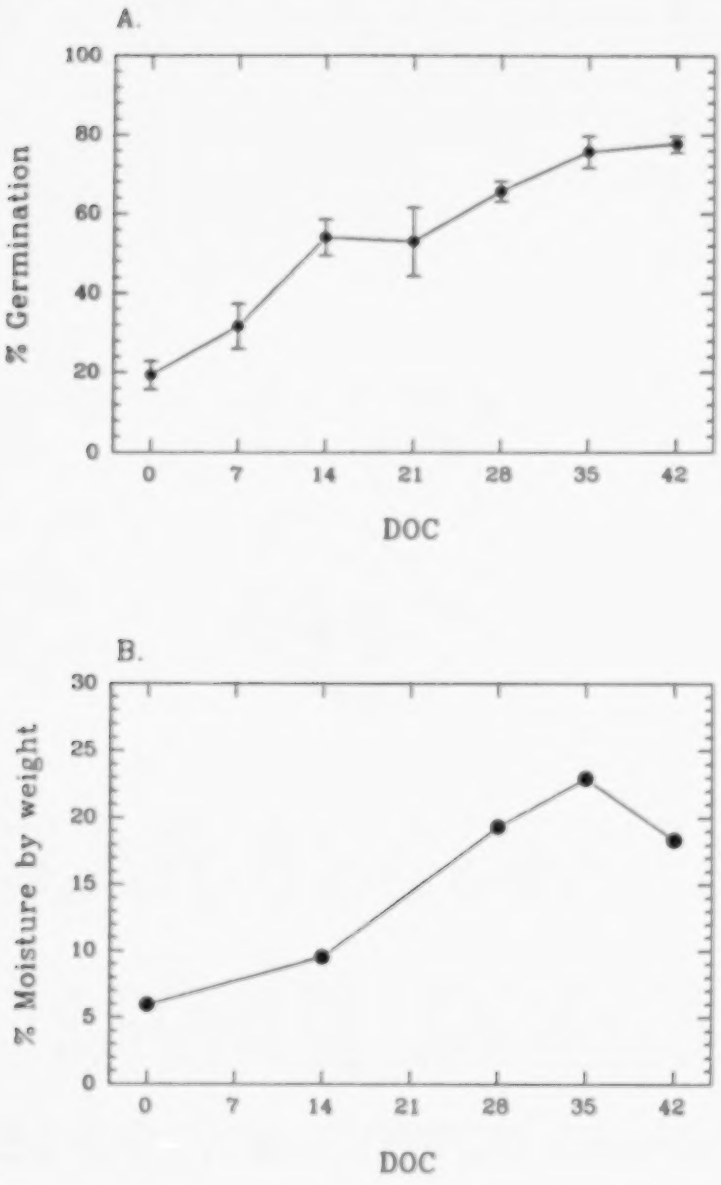


Figure 2 A. Percent germination through chilling treatment. Each data point is a mean of 3 determinations ±SE.
B. Percent moisture content per gram fresh weight through chilling treatment. Data points indicate the mean of 3 determinations.

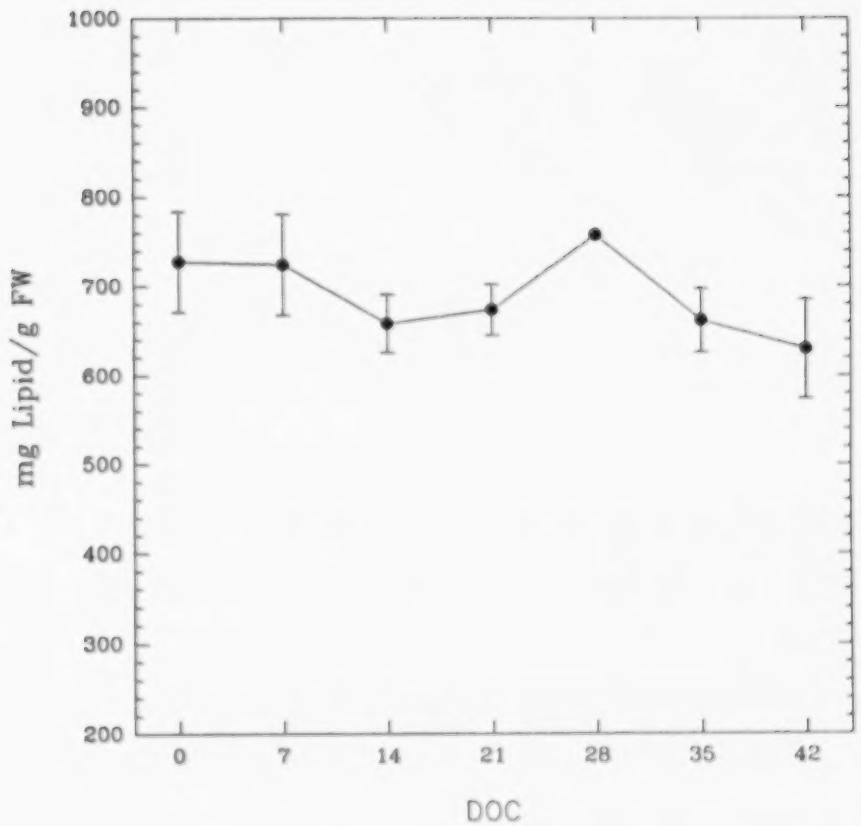


Figure 3 Lipid content of megagametophytes through chilling treatment measured as mg lipid per g fresh weight (FW). Each data point is a mean of 2 determinations ±SE.

fresh weight in the mature seed and 630±56 mg per gram fresh weight by 42 DOC.

The activity in cell-free extracts of malate synthase, an enzyme of the glyoxylate cycle, was assayed throughout chilling treatment. The results are shown in Fig. 4. Figure 4 shows that in the mature seed malate synthase activity was very low on day 0 on a per megagametophyte and on a per µg protein basis. There was only a slight increase in the activity of malate synthase through chilling treatment. At the highest point (28 DOC) the activity was 0.02 Units per µg protein and 6.5±1.3 Units per megagametophyte. For comparison, malate synthase activity was measured post-germination and early seedling growth (Fig. 5). From the end of chilling treatment, 42 DOC, up to radicle emergence at 4 DAI, there was an increase in malate synthase activity of 0.2 Units per µg protein and 38.0±2.7 Units per megagametophyte. At 4 DAI the malate synthase activity was 0.2 Units per µg protein and 43.2±5.1 Units per megagametophyte. This activity increased rapidly during early seedling growth and reached its highest point by 11 DAI (0.5 Units per µg protein and 197.3±7.1 Units per megagametophyte). The activity measured through germination and early seedling growth far exceeded the amount of activity shown through days of chilling. On a per µg of protein basis maximum malate synthase activity was 26 times greater than that during chilling treatment; the maximum malate synthase activity on a per megagametophyte basis was 30 times greater.

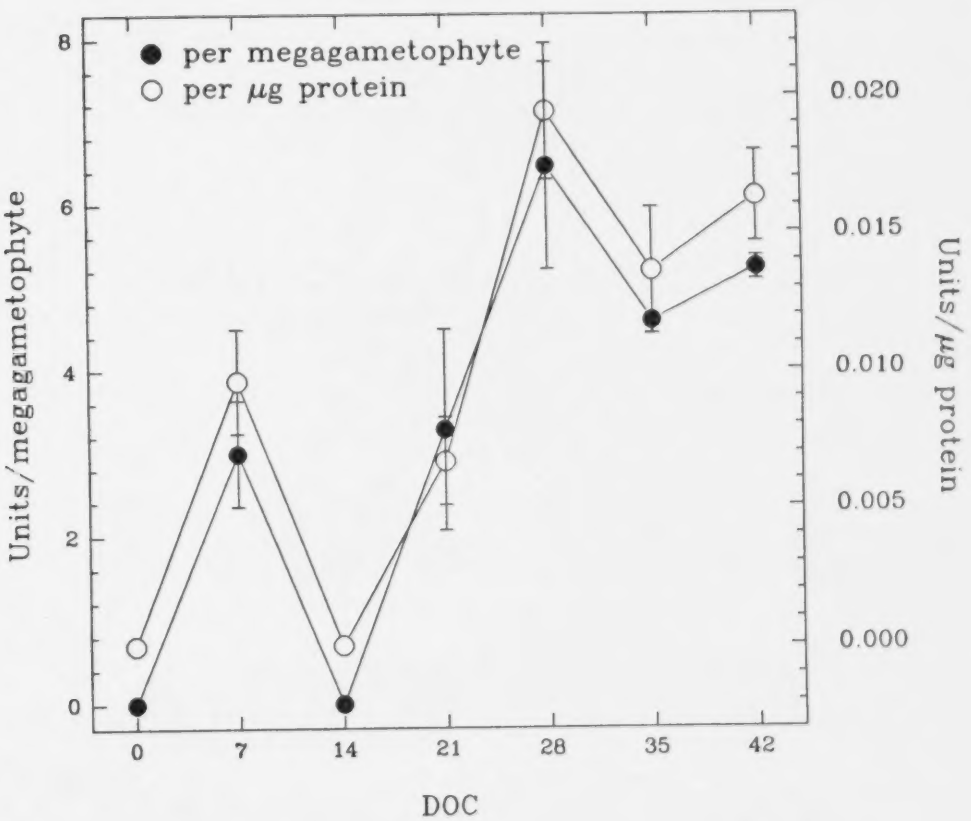


Figure 4 Malate synthase activity measured as Units per megagametophyte and Units per μg protein through chilling treatment. Data points are the mean of 3 determinations ±SE.

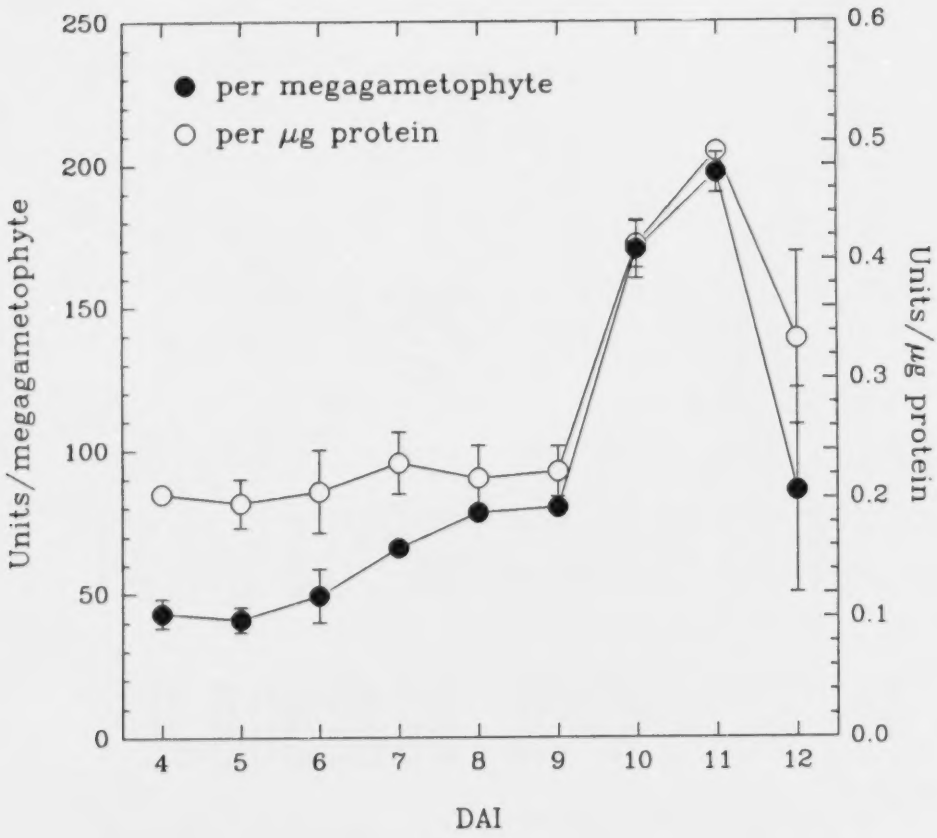


Figure 5 Malate synthase activity measured as Units per megagametophyte and Units per μg protein at the end of germination (4 DAI) and early seedling growth. Data points show the mean of 3 determinations ±SE.

3.4 Nucleic acids

Nucleic acid content was measured throughout the chilling period. No significant change in the total nucleic acid, DNA and RNA content of either embryo extracts (Fig. 6A) or megagametophyte extracts (Fig. 6B) was observed between 7 DOC and 42 DOC. The embryo extracts showed, at 7 DOC, total nucleic acid at 70.9±1.9 μg, DNA at 11.2 ±0.4 μg and RNA at 59.7±1.6 μg per embryo. By 42 DOC the values were very similar (75.0±3.5, 19.9±0.6 and 55.1±2.9 μg per embryo for total nucleic acids, DNA and RNA, respectively). The extracts of the megagametophyte showed, at 7 DOC, total nucleic acid at 89.5±3.6 μg, DNA at 12.8±1.1 μg and RNA 76.7±4.5 μg per megagametophyte. By 42 DOC the values are 56.5±3.9, 14.2±1.7, and 42.3±3.6 μg per megagametophyte of total nucleic acids, DNA and RNA, respectively. Between 0 and 7 DOC there was an apparent increase in total nucleic acid and RNA content. The embryo showed an increase from 34.3±5.1 and 27.8±4.3 μg per embryo to 70.9±1.9 and 59.7±1.6 μg per embryo in total nucleic acids and RNA, respectively. The megagametophyte increased in total nucleic acids and RNA from 47.8±6.6 and 36.0±5.4 μg per megagametophyte to 89.5±3.6 and 76.7±4.5 μg per megagametophyte respectively. Although this assay exhibits that DNA is remaining relatively constant through chilling treatment, it does not reflect changes that may be occurring in the individual populations of RNA.

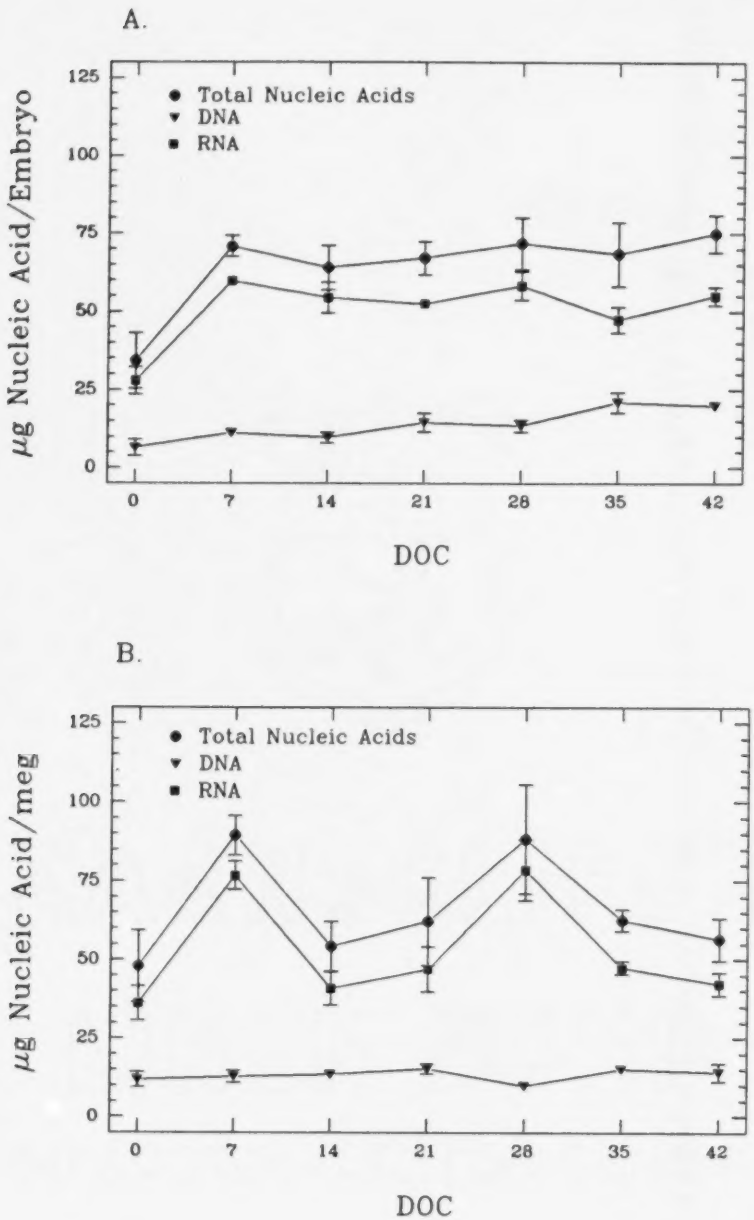


Figure 6 Nucleic acid content measured as μg of nucleic acid per embryo (A) and megagametophyte (B) through chilling treatment. RNA content was obtained by subtracting DNA from the total nucleic acid fraction. Each data point is a mean of 3 determinations ±SE.

3.5 Changes in proteins through chilling treatment

The quantity of total proteins in embryos and megagametophytes (Figs 7A and 7B, respectively), both buffer-soluble and insoluble, were compared throughout chilling treatment. There were only slight differences in the amounts of either buffer-soluble or insoluble proteins during the chilling period. Buffer-soluble proteins in the embryo ranged from 79.5±12.4 μg per embryo at 0 DOC to 115.6±6.4 μg per embryo at 42 DOC (Fig 7A). Buffer-insoluble proteins in the embryo ranged from 93.8±5.3 μg to 173.1±1.3 μg per embryo from 0 to 42 DOC (Fig. 7A). The buffer-soluble proteins of the megagametophyte extracts at 0 DOC were 134.3±8.7 μg and at 42 DOC 218.1±38.9 μg of protein per megagametophyte (Fig. 7B). They were approximately 2 times greater in quantity than the embryo extracts. The buffer-insoluble proteins ranged from 938.8±19.4 μg at 0 DOC to 2239.2±94.8 μg per megagametophyte at 42 DOC (Fig. 7B).

To determine if the concentration of specific proteins within the buffer-soluble and insoluble extracts were changing during chilling, the extracts were separated by SDS-PAGE. Coomassie blue stained gels are shown in Figs. 8 and 9. The figures depict that the protein profiles of both the buffer-soluble and insoluble extracts were very similar through chilling treatment. This can be seen by comparing the intensity of each protein band from 0 through to 42 DOC.

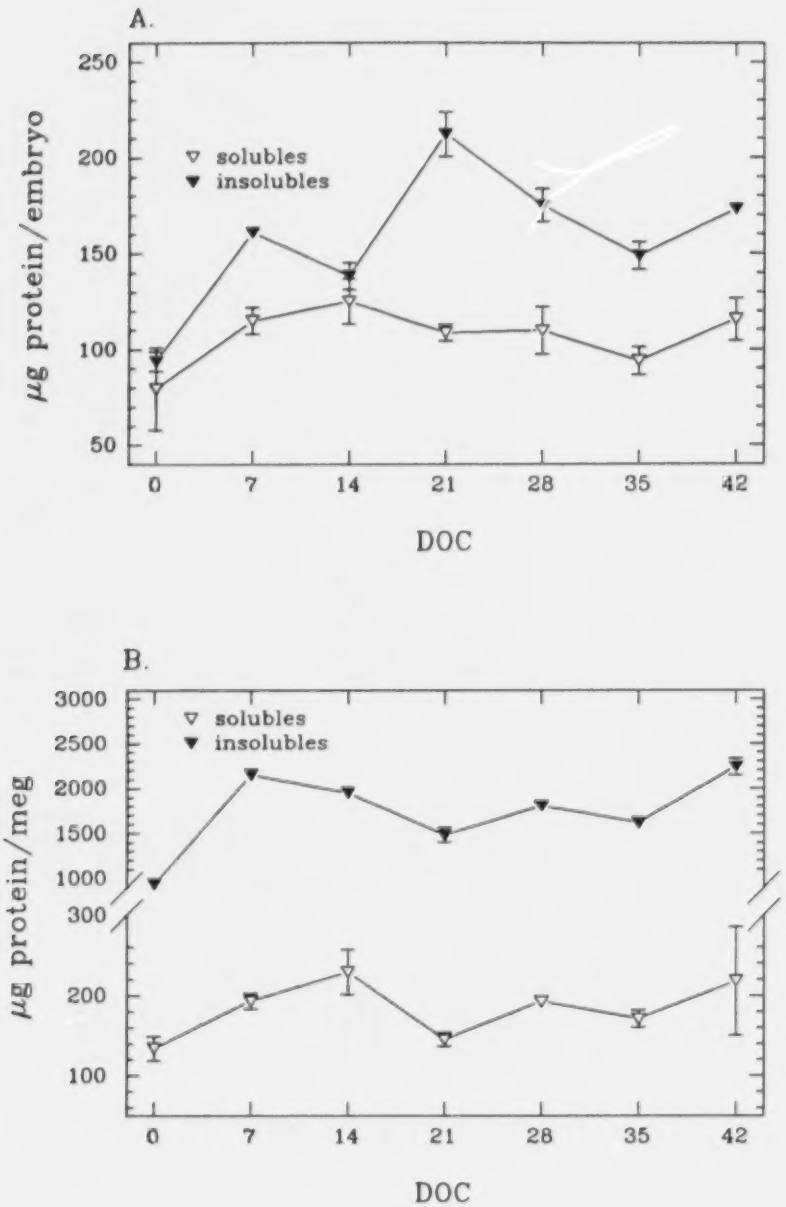


Figure 7 Protein quantitation through chilling treatment measured as µg of protein per embryo (A) and per megagametophyte (B). Each data point is a mean of 3 determinations ±SE.

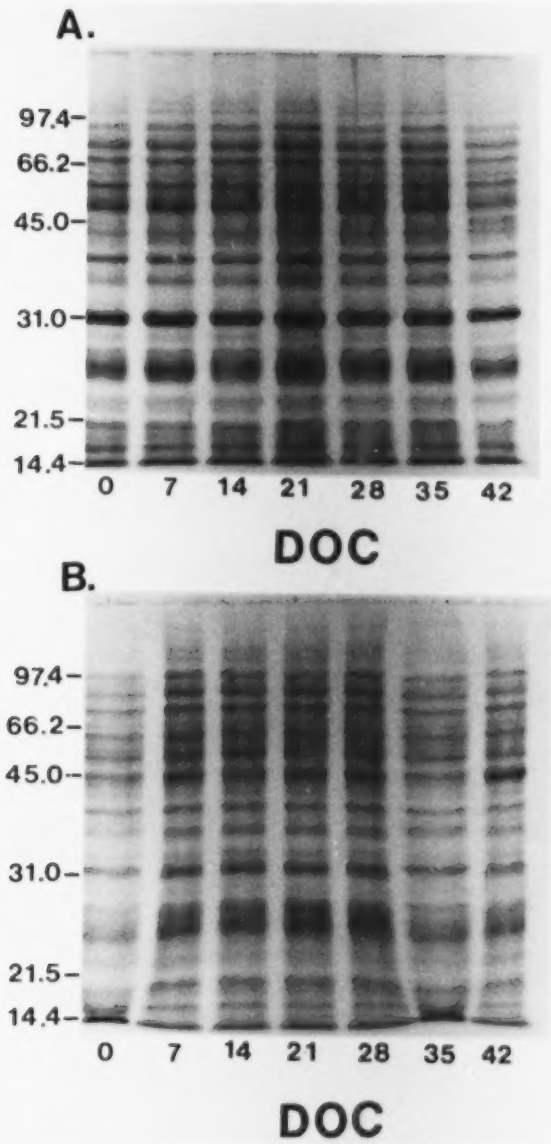


Figure 8 Coomassie stained SDS-PAGE gels showing the soluble protein profile of megagametophyte extracts (A) and embryo extracts (B) through chilling treatment. There were 10 seed parts in each extraction and 15 μg of protein in a 15 μL volume loaded per lane.

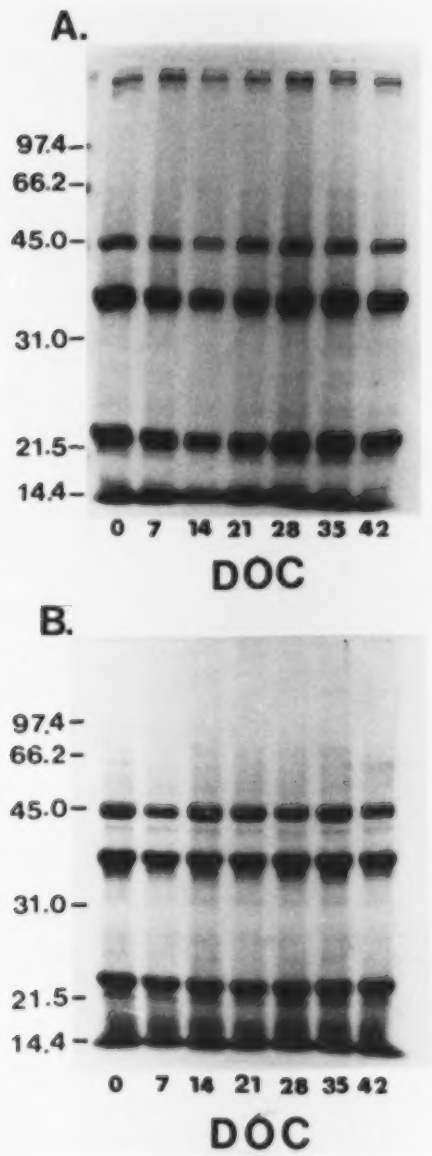


Figure 9 Coomassie stained, reduced SDS-PAGE gels showing the insoluble protein profiles of megagametophyte extracts (A) and embryo extracts (B). There were 10 seed parts in each extraction and 5 μg of protein in a 10 μL volume loaded per lane.

3.6 *In vivo* protein synthesis

While performing *in vivo* labeling experiments through chilling treatment, it appeared that the embryo had the ability to incorporate ^{35}S-methionine into protein at a much greater rate than the megagametophyte at each DOC sampled. Figure 10 shows the result of a typical labeling experiment comparing the embryo extracts with the megagametophyte extracts. Incorporation of label was determined by TCA precipitation. There was an 18 times greater incorporation into protein in the embryo compared to the megagametophyte at 0 DOC and a 2 times greater rate at 42 DOC. Through chilling there does appear to be an overall increase in the ability for embryos and megagametophytes to incorporate ^{35}S-methionine into protein.

To determine if the synthesis of specific proteins was changing during chilling treatment, megagametophytes and embryos were removed and incubated with ^{35}S-methionine to label the newly synthesized proteins. Proteins in the soluble extracts were separated by SDS-PAGE and labeled proteins were identified by fluorography. Figure 11 shows representative fluorographs. In the embryo, protein synthesis is similar throughout chilling treatment (Fig. 11A). The absence of protein bands on the gels indicates that no new proteins were being synthesized, but there were some bands disappearing in the low molecular weight region, i.e. 15 to 40 kDa, indicating that the synthesis of these proteins was decreasing throughout chilling treatment.

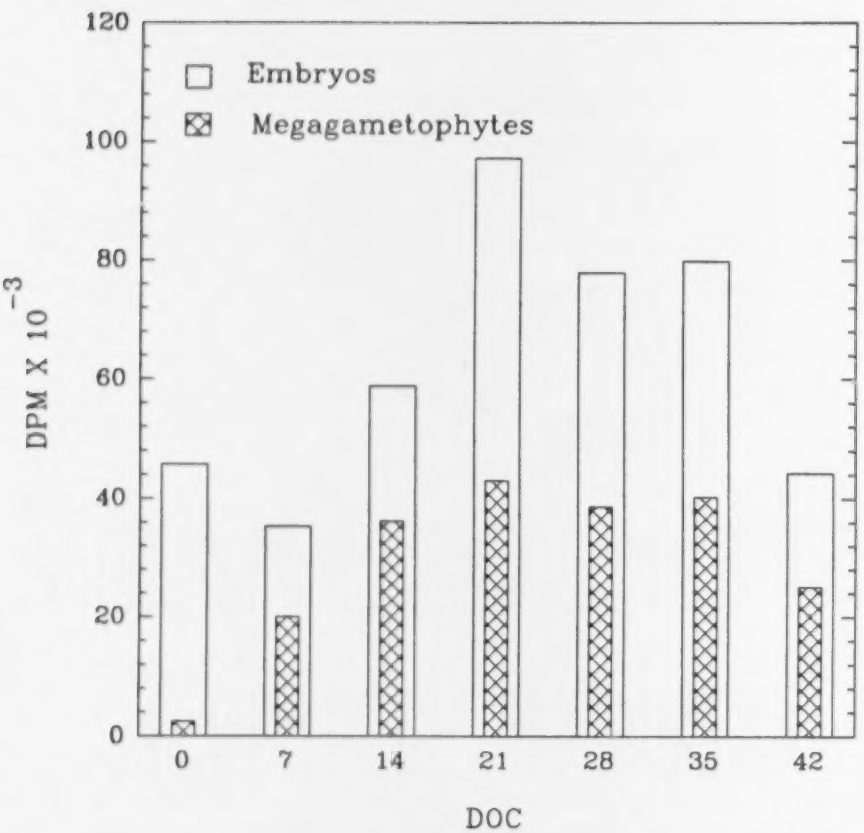


Figure 10 Bar chart of a typical labelling experiment showing the difference in incorporation of ^{35}S-methionine into protein in the embryo compared with the megagametophyte as determined by TCA precipitation.

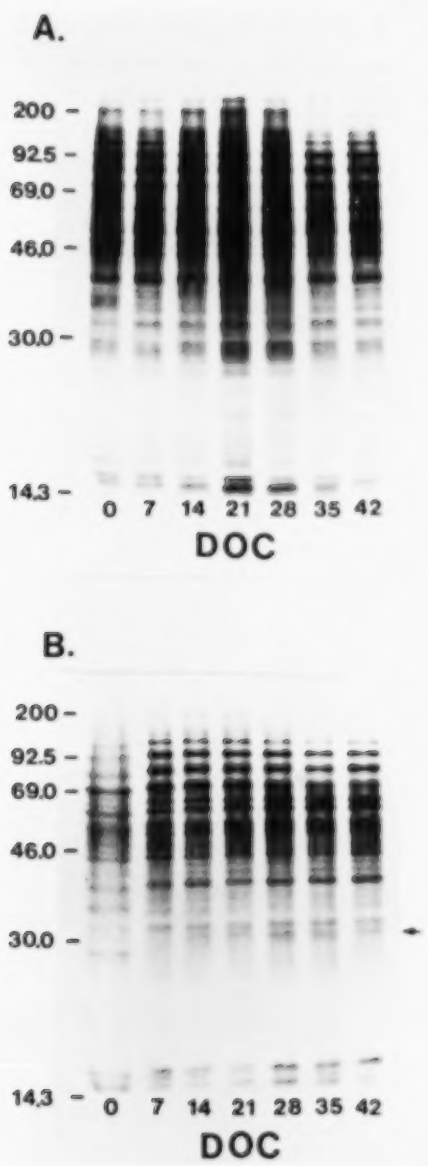


Figure 11 A. Fluorograph of gel following SDS-PAGE showing incorporation of ^{35}S-methionine into new proteins by embryonic tissue through chilling treatment. Numbers to the left of the gels indicate ^{14}C-methylated standards. The lanes were loaded with 2500 dpm in 10 μL.
B. Fluorograph of a gel following SDS-PAGE showing incorporation of ^{35}S-methionine into new proteins by megagametophytic tissue through chilling treatment. The lanes were loaded with 2500 dpm in 10 μL. Arrow to the right of the gel indicates the appearance of a new protein, see text for details.

A typical one dimensional fluorograph of megagametophyte extracts (Fig. 11B) also showed the decrease in synthesis of some low molecular weight proteins, as in the embryo extract fluorographs. However, there appeared to be an additional change in the pattern of protein synthesis that occurred between 14 and 21 DOC. Specifically, there was the synthesis of proteins with molecular masses of 31 kDa (indicated by an arrow at the right hand side of the fluorograph). These appeared faintly at 14 DOC, their synthesis increased until 28 DOC and then started to decrease between 35 and 42 DOC.

To further examine changes in synthesis of specific proteins occurring during chilling, soluble extracts of labeled proteins were separated by IEF/SDS-PAGE (Fig. 12). The 31 kDa proteins of the megagametophyte extracts indicated on the SDS-PAGE fluorograph were present on the acidic end of the two-dimensional fluorograph (encircled). These proteins appeared at 14 DOC, increased up until 28 DOC and decreased between 35 and 42 DOC as on the one-dimensional fluorograph. In addition to these proteins, other newly synthesized proteins (encircled) at the acidic end included proteins around the 77.6 kDa mass, in the neutral portion of the gel 14.8 kDa and 16.9 kDa masses and at the basic end of the gel 67.6 kDa. These proteins also began to appear between 14 and 21 DOC and stopped being synthesized after 28 DOC. Other proteins (indicated by boxes) began to be synthesized during late chilling (21 DOC) and included proteins in the acidic 64.6 kDa domain. For orientation, constitutive proteins are indicated by encompassing brackets with a tick.

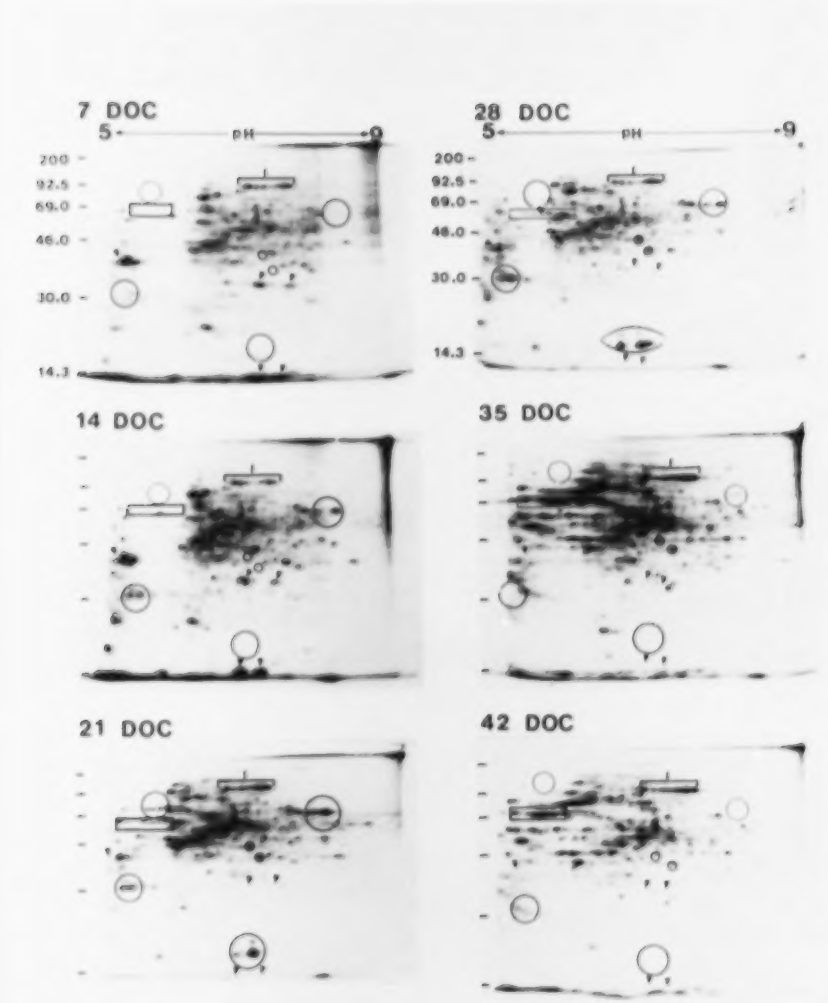


Figure 12 Fluorograph of gel following IEF/SDS-PAGE showing incorporation of ^{35}S-methionine into new proteins by megagametophytic tissue through chilling treatment. The pH range is indicated at the top and the ^{14}C-methylated standards are to the left of the gels. The encompassing brackets with ticks are indicating constitutively expressed proteins, the arrowheads indicate proteins disappearing with time. The circles indicate proteins that are peaking during cold treatment, the rectangles indicate proteins arising during late chilling treatment. In each case 14,000 dpm in 10 μL was loaded in the first dimension.

In contrast, further analysis of embryo extracts using IEF/SDS-PAGE (Fig. 13) reflected what was found on the one dimensional fluorograph, that the synthesis of some low molecular weight proteins decreased during chilling treatment. These proteins, indicated by arrows on the Figure, show most synthesis at 7 DOC, this synthesis decreases through 14 and 21 DOC and ceases by 28 DOC. Also indicated on the two-dimensional fluorograph are proteins that are synthesized at constant rates (encompassing bracket with a tick) through chilling treatment. No change in new protein synthesis by the embryo during chilling, similar to that seen for the megagametophyte, was detected using IEF/SDS-PAGE.

3.7 Isolated embryo experiments

3.7.1 Germination and Growth

To determine the type of dormancy in loblolly pine seeds, embryos were isolated from mature unstratified seeds and placed on unsupplemented water agar. They were non-dormant, germinating at 30°C, as indicated by elongation of the hypocotyl and greening of the cotyledons (Fig. 14). The isolated embryos grew, on average, a length of 13 mm after 4 days at 30°C.

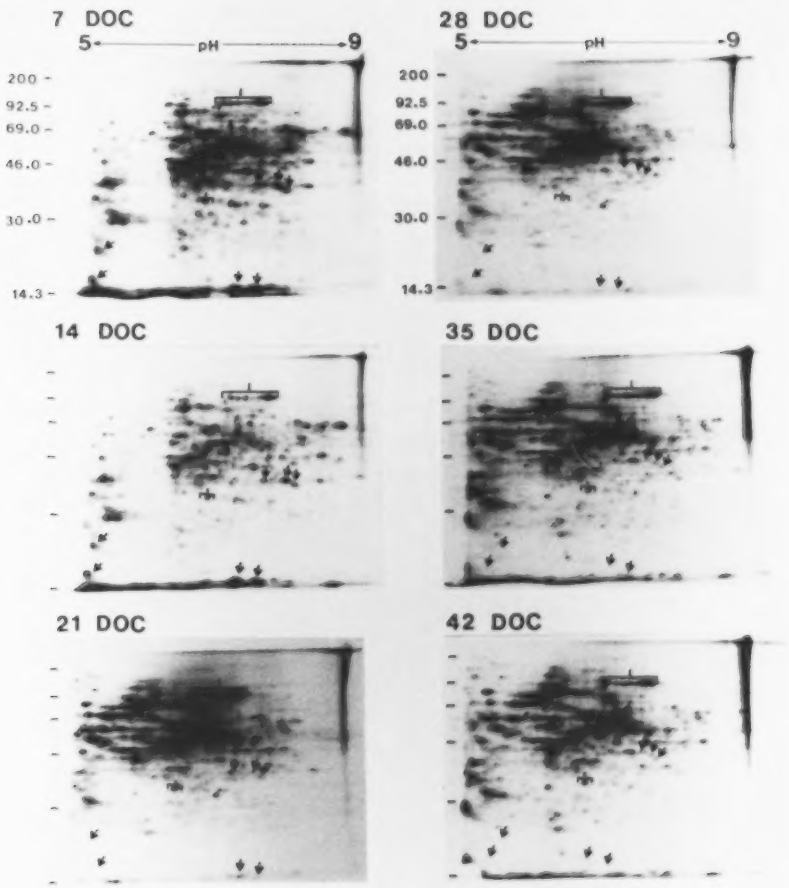


Figure 13 Fluorograph of a gel following IEF/SDS-PAGE showing incorporation of ^{35}S-methionine into new proteins by embryonic tissue through chilling treatment. Other labels and gel loading are as per Fig. 12.

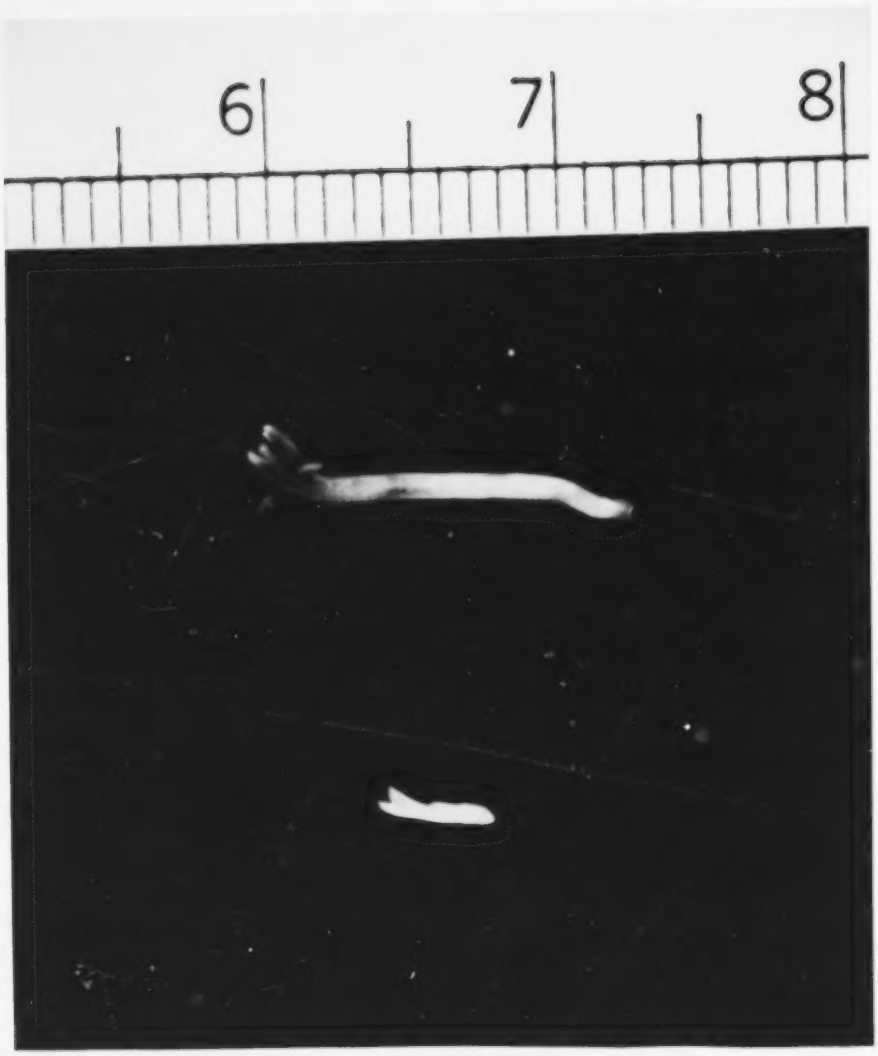


Figure 14 Print of germinated isolated embryo beside a freshly excised embryo, both from mature seed. Excised embryos were placed on 3% water agar and set in an illuminated germinator for 5 days at 30°C. See text for details.

3.7.2 Germination and Embryo Proteins

To further understand the biochemical nature of embryos isolated from mature seed, they were germinated at 30°C and their development compared with germinating embryos from fully stratified, i.e. 42 DOC, seed. Loblolly pine seed embryos contain several storage proteins that are rapidly hydrolyzed following imbibition at 30°C (Groome et al., 1991). The rate of mobilization of these reserves in embryos from mature seed was compared to that occurring in embryos from stratified seed. There are three major storage proteins in loblolly pine seed embryos (Groome et al., 1991). They have molecular masses of 60 kDa, 47 kDa and 38 kDa respectively (Fig. 15 M., lane 0; Fig 15 S., lane 0). Under reducing conditions, the 60 kDa protein separates into its component peptides with molecular masses of 37 kDa and 23 kDa. This can be seen in Fig 16 M., lane 0 and Fig 16 S., lane 0. During a five day growth period, both embryo types mobilized these three stored protein reserves in similar fashions (see for example, Fig 16, M. & S.). To further evaluate the rate of disappearance of these storage proteins following imbibition at 30°C, the protein amount at each DOC was determined using scanning densitometry. Figure 17 shows the result for the 47 kDa storage protein. Linear regression of the rate of breakdown of this protein was comparable in both mature, unstratified embryos and fully stratified embryos. Similar results were obtained for the two other major embryo storage proteins. SDS-PAGE and scanning densitometry have indicated that both embryo types have the ability to mobilize their protein reserves.

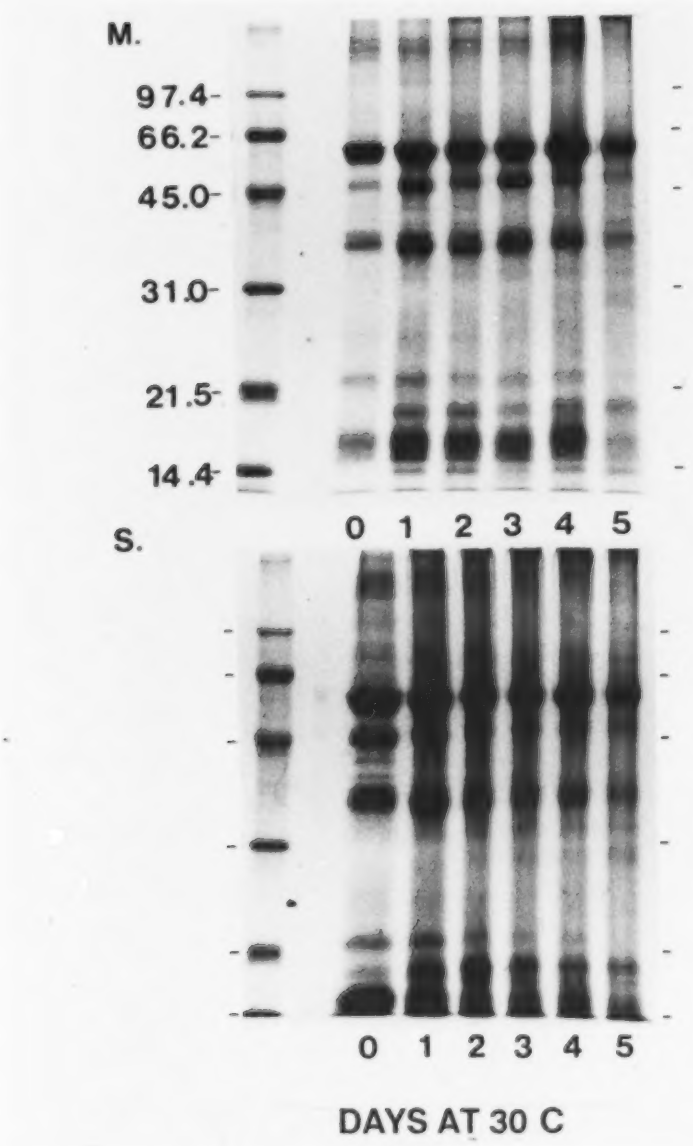


Figure 15 Coomassie stained SDS-PAGE showing unreduced protein breakdown products of isolated embryos from mature seed (M) compared to isolated embryos of stratified seed (S) through days at 30°C. In each lane 5 μg of protein in a 10 μL volume was loaded. Protein molecular mass standards are indicated at the left side of the gel.

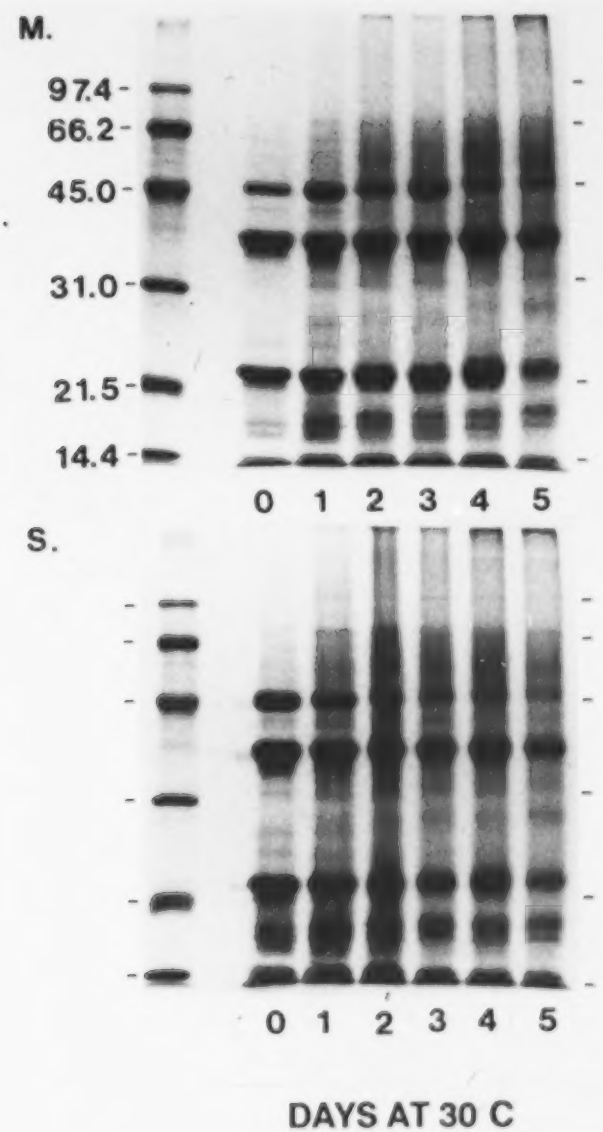


Figure 16 Coomassie stained SDS-PAGE showing reduced protein breakdown products of isolated embryos from mature seed (M) compared to isolated embryos of stratified seed (S) through days at 30°C. In each lane 5 μg of protein in a 10 μL volume was loaded. Labels as for Fig. 15.

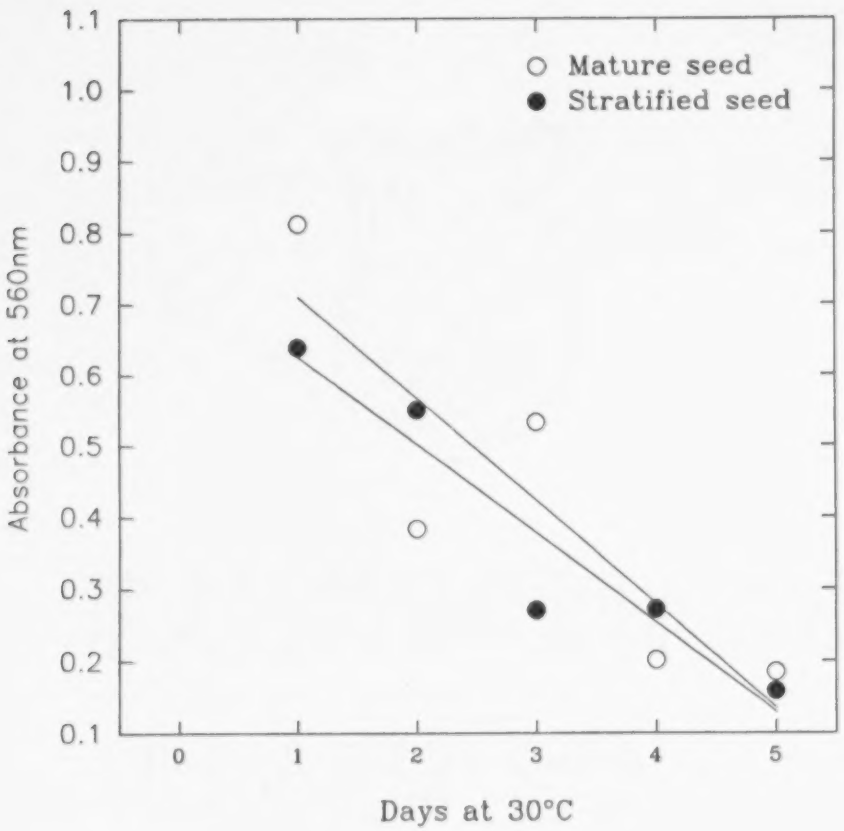


Figure 17 Linear regression of data of the results of scaning densitometry showing breakdown of the 47 kDa protein at an Absorbance of 560 nm. Gel scanning was performed on the reduced Coomassie stained SDS-PAGE gels of the mature (M) and stratified (S) isolated embryos of Fig. 16.

3.7.3 Germination and *De Novo* Protein Synthesis

To further compare the mature and stratified embryos, their ability to synthesize proteins *de novo* was compared. Embryos were removed following incubation at 30°C and proteins were incubated with ^{35}S-methionine. The buffer-soluble, labelled proteins were separated by SDS-PAGE and fluorographs were obtained. The representative fluorographs are shown in Figure 18 (M and S). No significant differences between embryo types were observed. They each have the ability to synthesize the same sets of proteins as determined by the same protein profile bands on the fluorographs. The patterns of protein synthesis following imbibition at 30°C for the two embryo types were further characterized by using IEF/SDS-PAGE followed by fluorography (Figs. 19 and 20, respectively). The patterns of protein synthesis were the same. For orientation, constitutive proteins are shown by an encompassing bracket with a tick. To further substantiate that both embryo types were synthesizing the same sets of proteins, the fluorographs were placed one on top of the other over a light-box. The dots (which indicated a new protein) on the mature embryo fluorograph were superimposable on the dots of the stratified embryo fluorograph.

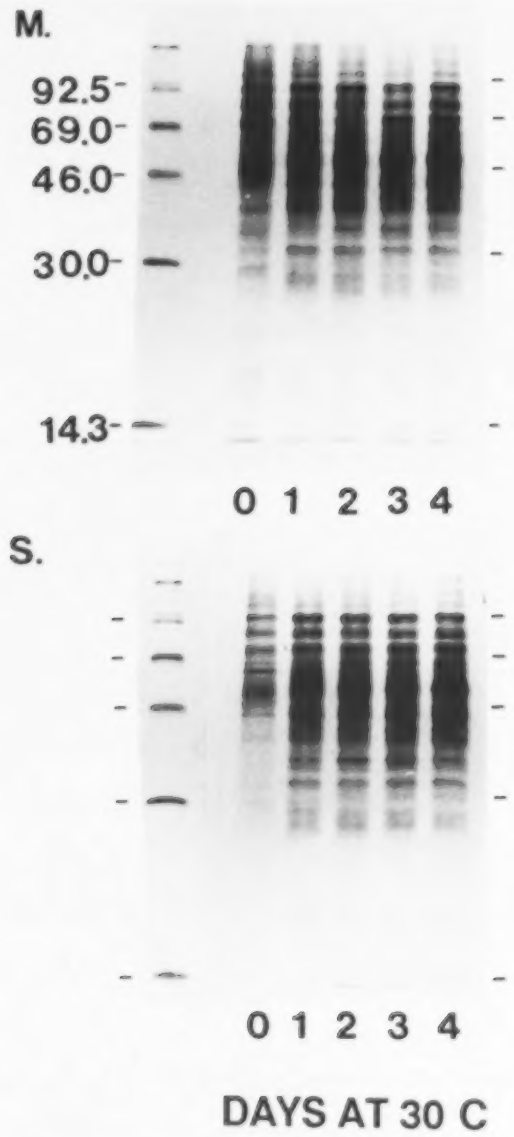


Figure 18 Fluorograph of a gel following SDS-PAGE showing the incorporation of ^{35}S-methionine into new proteins in both mature (M) and stratified (S) isolated embryos through days at 30°C. In each lane 20,000 dpm in a 10 µL volume was loaded.

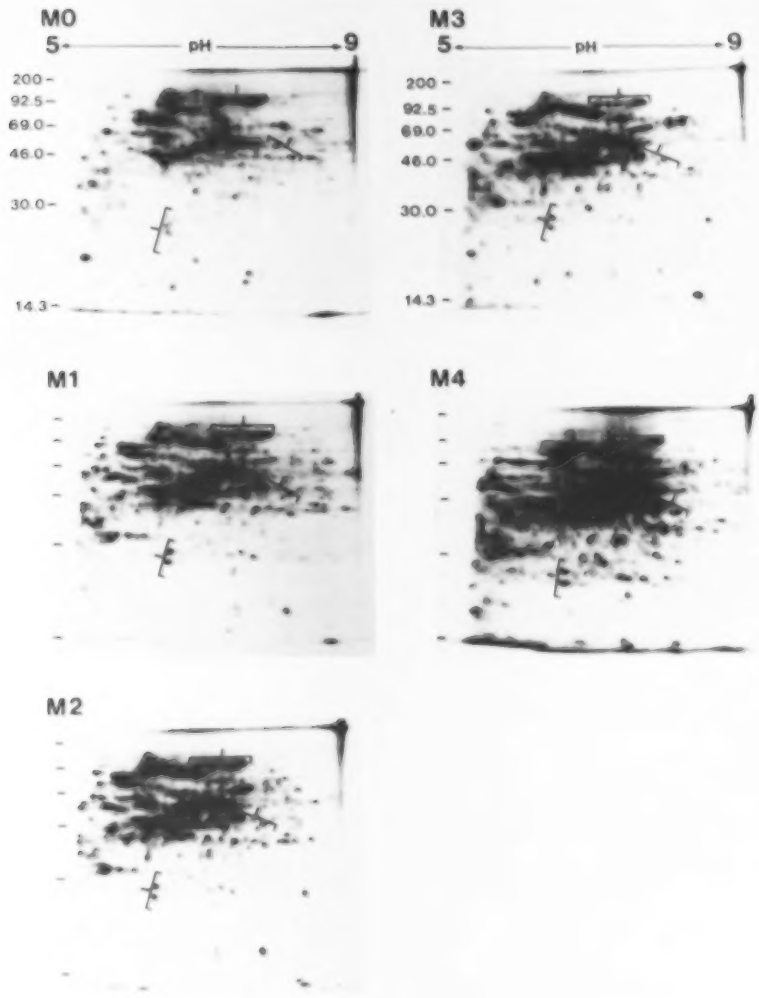


Figure 19 Fluorograph of a gel following IEF/SDS-PAGE showing the incorporation of ^{35}S-methionine into new proteins in isolated embryos of mature (M) seed through days at 30°C. In each case 40,000 dpm in a 10 µL volume was loaded in the first dimension.

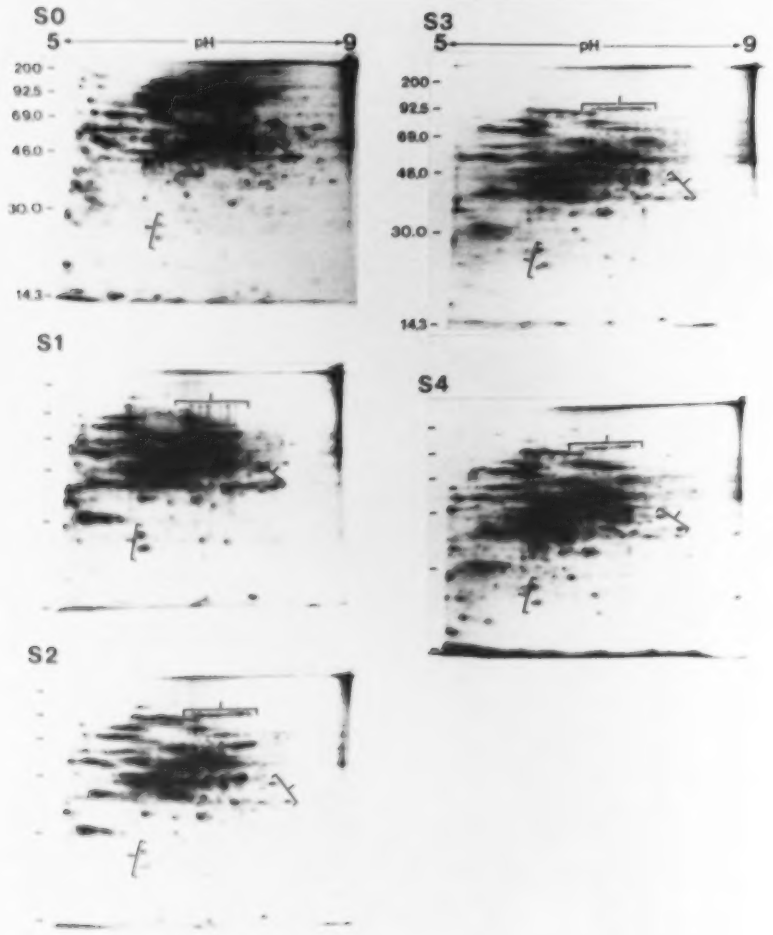


Figure 20 Fluorograph of a gel following IEF/SDS-PAGE showing the incorporation of ^{35}S-methionine into new proteins in isolated embryos of stratified (S) seed through days at 30°C. Gels were loaded as described in Fig. 19.

3.8 Sequential removal of tissue experiments

3.8.1 *Pinus taeda* Seed Anatomy

The anatomy of a mature loblolly pine seed is shown in Figure 21. The embryo lies in a fluid-filled cavity, this is often referred to as a corrosion cavity. Surrounding the cavity is the megagametophyte. The megagametophyte is comprised of storage parenchyma cells which house the majority of the storage reserves; lipids and proteins. On the micropylar end of the seed is a structure called the nucellar cap. It is thought that the nucellar cap protects what once was the micropyle. The three-layered integument makes up the single seed coat of the pine seed. The inner layer of the integument (indicated as a dashed line) is a thin, papery, membranous structure that is in close proximity to the epidermal layer of the megagametophyte. Next to the inner layer, lies the stony layers of the middle and outer integument (shown as hatched areas) which from this point forward will be referred to as the 'hard' seed coat. These two layers of integument are indistinguishable from each other when dissecting the seed (Owens and Molder, 1977).

3.8.2 Tissue Removal and Seed Germination

Loblolly pine seed embryo is nondormant but it fails to germinate *in situ*. To determine which seed tissue was imposing dormancy the components of the seed surrounding the embryo were

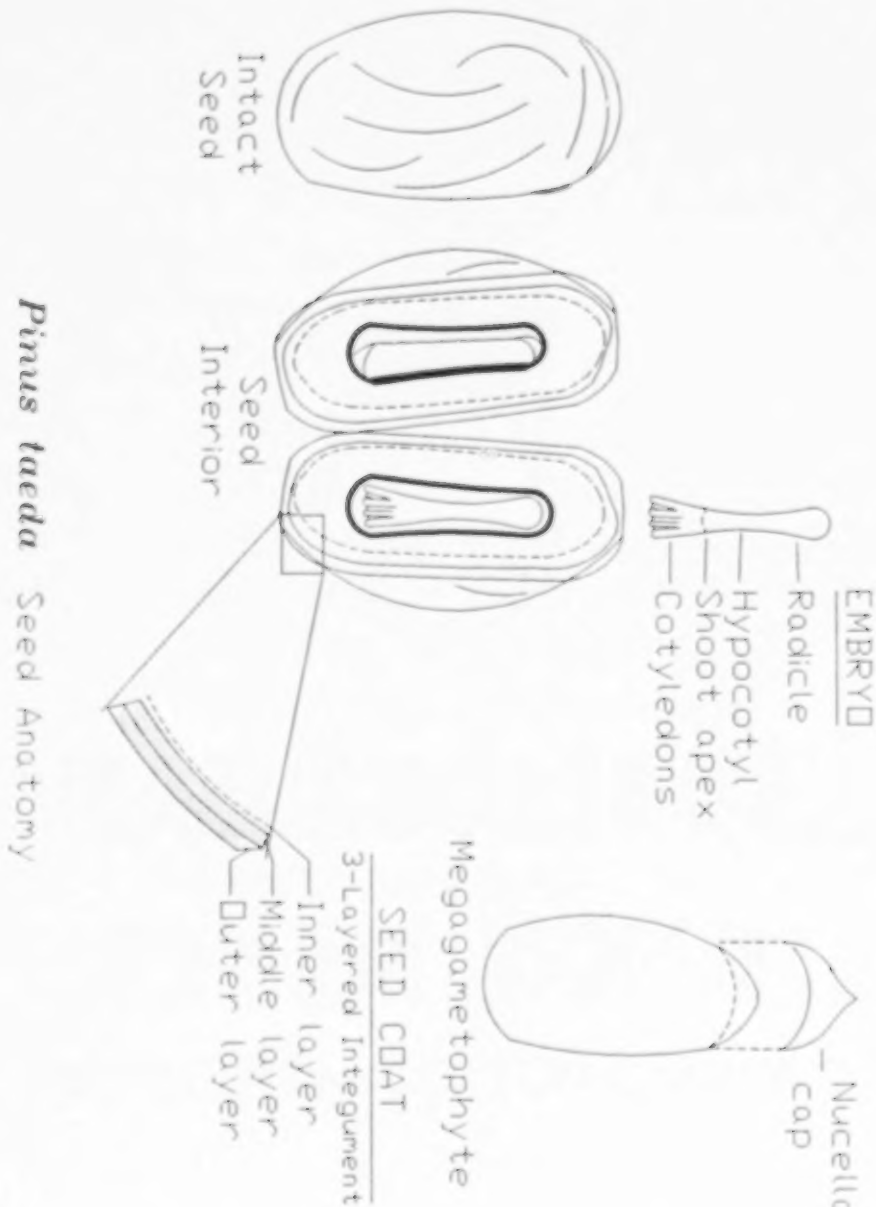


Figure 21 Diagrammatic representation of loblolly pine seed anatomy. See text for details.

sequentially removed and embryo germinability was assessed. All seeds germinated when the hard seed coat was removed. This was also true for the inner layer of integument of the seed coat with or without the nucellar cap. However, seeds with all tissues external to the megagametophyte removed did not germinate as well on wet filter paper as they did on water agar (Fig 22). The whole seed control showed a background germination of approximately 20% (Fig. 22A). There was an increase in germination on wet filter paper when the integuments were removed (Fig. 22B). However, 100% germination occurred when the seeds *sans* integuments were placed on water agar (Fig. 22C). Isolated embryos also showed 100% germination when placed on unsupplemented water agar (Fig. 14).

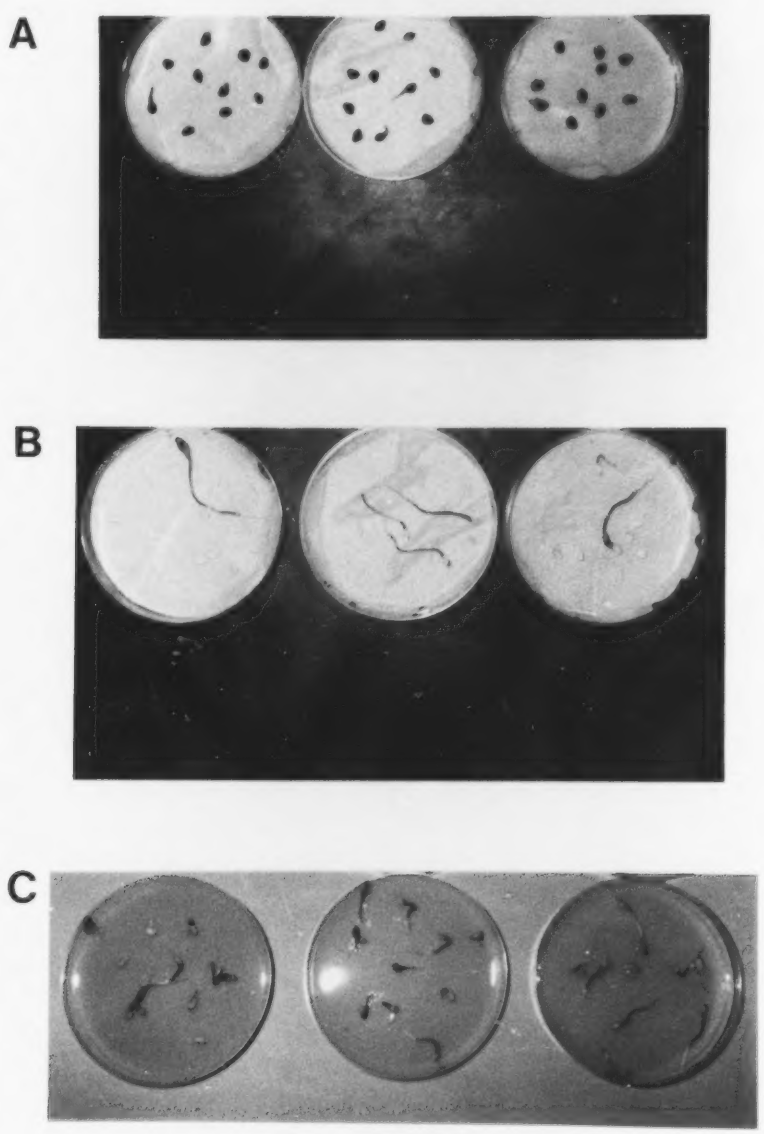


Figure 22 Barriers to germination. All seeds or seed-parts were placed for 5 days at 30°C in an illuminated germinator. A. Control, of whole seed on wet, sterile filter paper. B. Seeds without integuments and nucellar cap on wet, sterile filter paper. C. Seeds without integuments and nucellar cap on 3% water agar.

IV DISCUSSION

Portions of this thesis dealing with changes in protein synthesis during the breaking of dormancy in loblolly pine have been submitted to Physiologia Plantarum (Schneider and Gifford, May 1993).

4.1 Trends during chilling treatment

Biochemical and molecular changes occurring as dormancy is lost have not been studied extensively before in pine seeds. Past research has focused more on empirical methods for breaking dormancy (Barnett, 1972; Barnett, 1976; Carpita et al., 1983; Bewley and Black, 1985; Cohn, 1987). In this study, breaking of dormancy in loblolly pine seed was studied with specific emphasis on changes occurring during chilling treatment. It is plausible to assume that whatever transition occurs that changes the seed from the dormant state to the nondormant state would occur during chilling treatment. Therefore, this study looked for those changes. Specifically, changes in percent germination, moisture content, lipid breakdown and glyoxylate cycle activity, nucleic acid and protein content and synthesis were monitored during chilling treatment.

4.1.1 Germination Ability and Water Uptake

The percent germination results using bleach sterilization showed loblolly germinating at an increasing rate that corresponded

with DOC. After 42 DOC the seeds showed a maximum germination of 78±2%. This indicates that loblolly pine seed requires a chilling treatment for increased and uniform germination. Moisture content in the whole seed increased to 23% per gram of fresh weight during chilling treatment as would be expected for mature seed only has a 6% water content by weight. The increases in percent germination and water content correlate well with the time spent in chilling treatment. The degree of hydration would also affect the metabolic activity during the transition from dormancy to non dormancy (Ching, 1966).

4.1.2 Lipids and Glyoxysomal Activity

If a seed is truly dormant, high metabolic activity during the dormancy period would not be expected. Therefore, storage reserves such as lipids and proteins should not be broken down. Lipids, the major storage reserve in loblolly pine seed (Groome et al., 1991), were measured through chilling treatment and remained unchanged. If lipid breakdown were occurring it would be through the β-oxidation and the products of this would be subsequently metabolized in the glyoxysome, (see Methods and Materials). The amount of malate synthase activity shown through chilling treatment was very low compared to the activity shown post-germination. It was concluded that there was no glyoxysomal activity through chilling treatment. Two other enzymes of the glyoxylate cycle, isocitrate lyase and catalase, have been monitored in loblolly pine in the mature seed and in fully stratified, ungerminated seed (Groome, Mullen and Gifford,

unpublished results). No activity of either enzyme was detected. Why then is there some, albeit low, malate synthase activity during chilling treatment? Research into the enzymes of the glyoxylate cycle showed that the proportion of malate synthase that is formed *de novo* may vary considerably. Miernyk et al. (1979) studied glyoxylate cycle enzymes in cotton, castor bean and 18 other oilseeds. They found that malate synthase from cotton is stored in the dry seed, while in castor bean both malate synthase and glyoxysomal malate dehydrogenase are formed *de novo* following imbibition. Furthermore, malate synthase activity was measureable in all seed types examined, whereas another glyoxylate cycle enzyme, isocitrate lyase, could not be detected in any. It is now generally accepted that synthesis of malate synthase during seed development is universal among oilseeds in the absence of glyoxylate-cycle-associated isocitrate lyase activity. This enzyme may be one of the stored proteins in loblolly pine seeds and it may be that once liberated in the cell-free system, its activity is expressed. The reason for the presence of low but significant malate synthase activity in the mature seed and during chilling treatment has yet to be determined.

4.1.3 Nucleic Acid Content

Ching (1966) found that the embryo of Douglas fir seed showed a slight increase in DNA content during stratification and a sharp increase at the radicle emerging stage. However, when nucleic acids were measured in megagametophytes and embryos of loblolly pine seeds through chilling treatment no significant changes in DNA

content were observed. This may be due to a lack of nuclear division occurring in the embryo i.e., the embryo *in situ* is still dormant through chilling and does not begin cell division until post-germination. This has yet to be determined. In contrast, total RNA content fluctuated during chilling treatment. While the present study did not evaluate specific RNA populations, the observed changes in total RNA occurring during chilling could relate to changes in the pattern of protein synthesis that were observed for both the embryo and the megagametophyte. The fluctuations in total RNA may be partially due to the extractability of nucleic acids from the mature seed embryos and megagametophytes. The mature seed embryo and megagametophyte are very brittle at the end of maturation drying. At this stage it is very difficult to separate the embryo from its surrounding tissues without compromising its integrity. At the same time, due to its brittle nature it is more difficult to macerate. After 7 days of chilling, however, the seed has hydrated enough to make tissue isolation and extraction much easier.

4.1.4 Protein Profiles

4.1.4.1 Quantitative changes in storage proteins through chilling treatment

It was once thought that the limited hydrolysis of storage reserves, specifically seed storage proteins, during chilling treatment was a requirement for germination (D.G.W. Edwards, Canadian Forestry Service, personal communication). This was not the case in

loblolly pine seeds. Quantitative changes in embryo or megagametophyte storage proteins were not observed. However, this does not reflect the changes in protein synthesis that occurred during chilling treatment.

4.1.4.2 *In vivo* protein synthesis

While storage protein hydrolysis did not occur during chilling treatment, protein synthesis did. To evaluate changes in patterns of synthesis of new protein occurring as dormancy is lost, proteins were labelled with 35S-methionine. Dure, et al. (1981), showed that radioactively labelled proteins were easily depicted using autoradiography; however, those same proteins were not picked up using Coomassie blue staining as they were not sufficiently abundant. In both embryos and megagametophytes most of the 35S-methionine labeled proteins appeared to be synthesized constitutively. The embryo extracts did not appear to synthesize any new proteins but rather stopped synthesizing the Lea-like proteins mentioned earlier. If RNA is present in the mature seed as a translatable message, then it is possible that new proteins that aid in the seed's transition from dormancy to nondormancy may arise during chilling treatment . For example, Lea mRNAs are conserved during seed maturation, translated for a brief period following rehydration, and then rapidly degraded (Dure et al., 1989). As well, Masumori, et al. (1992) found stored mRNA molecules in mature pine seeds indicating that they must have been synthesized during seed development and then stabilized before dormancy. They proposed that the mRNA is present

as some stabilized complex with ribonucleoproteins protecting the mRNA from digestion. If this occurs in loblolly pine seed, it would explain the seed's ability to quickly supply *de novo* proteins that may aid in breaking dormancy. Unlike embryo extracts, megagametophyte extracts showed a significant shift in the pattern of protein synthesis through chilling. On the SDS-PAGE fluorograph new proteins began to appear at 14 DOC and were prominent at 21 and 28 DOC. Further characterization using IEF/SDS-PAGE showed that these are acidic and approximately 31 kDa in molecular mass, based on mobility against 14C-methylated standards. Their presence may imply that the megagametophyte has an influence on the embryo. The new proteins could be markers of removal of a constraint that maintains the dormancy. This would account for the disappearance of proteins through chilling treatment. Increases in germinability shown in loblolly pine seed is not linear but rather coincides with changes in protein synthesis. Once the constraint is removed, new gene expression in the megagametophyte could be initiated, allowing germination to proceed. This hypothesis is supported by results obtained when the embryo is taken away from the environs of the megagametophyte and it readily germinates. As well, this group of proteins are not synthesized during germination; i.e. these are not part of the germination protein set (Groome et al., 1991).

4.2 Isolated Embryo Experiments

The embryo of mature loblolly pine seed is nondormant. When

isolated from the seed and placed in appropriate conditions it germinated. However, is it the same biochemically as an embryo from a fully stratified seed, i.e. does it have the same ability to mobilize its reserves and synthesize new proteins? The rate of mobilization of three storage reserve proteins in embryos from mature and stratified seed was compared. The breakdown products of the proteins were depicted on Coomassie stained gels and then quantified using scanning densitometry. The results showed the rates of mobilization of reserves in both embryo types was the same. These similarities were further substantiated when *in vivo* protein labeling experiments were done. By carefully comparing fluorographs, it was shown that both embryo types have the ability to synthesize the same sets of proteins. If the synthesis of specific proteins is needed to maintain embryo dormancy, these would have to be supplied by the megagametophyte. One hypothesis may be that, proteins synthesized by the megagametophyte would be exported to the embryo, via the corrosion cavity, where they would inhibit the germination gene set; the expression of which is an essential component of normal embryo germination and radicle growth (Groome et al., 1991). In this model, the termination of synthesis of these germination-inhibiting proteins would initiate the transition to nondormancy. As a further addendum to this model, it could be hypothesized that the newly synthesized 31 kDa proteins, seen at 14, 21 and 28 DOC, could be inducers of germination. They may act as a binding proteins to the inhibitor rendering it inactive, or, the proteins may confer tolerance to the embryo such that the inhibitor no longer exerts its influence.

4.3 Barriers to Germination

4.3.1 Imposed Dormancy

More than 80% of the embryos from mature, unstratified loblolly pine seed failed to germinate *in situ* when placed in appropriate conditions. When such embryos were removed from the seeds, they readily germinated *in vitro*. Thus, embryo dormancy in the mature unstratified seed is imposed. Egley (1989) defined seeds as "hard" if the seedcoat acted as a water-impermeable covering that prevented hydration of the seed thereby preventing germination until the permeability breaks down. Studies in pine have implicated the seedcoat as a factor in dormancy maintenance (Hoff, 1987; Pitel and Wang, 1985; Barnett, 1976; Larsen, 1925). The inner layer of the three-layered integument of the seedcoat (in the literature this is often referred to as the nucellus), which is situated between the middle layer of the integument and the megagametophyte (Fig. 21), has been described as restricting water absorption in sugar pine (Baron, 1978), oxygen transport in Jeffrey pine (Stone, 1957) and eastern white pine (Kozlowski and Gentile, 1959) and as a major site of dormancy in western white pine (Pitel and Wang, 1985; Hoff and Steinhoff, 1986; Hoff, 1987). The inner layer of integument and nucellar cap may act to control rather than impede water uptake in loblolly pine seeds. If the amount of water allowed to enter the megagametophyte is kept low, then this may prevent hypoxic conditions arising within the seed. This is somewhat substantiated by results obtained involving removal of tissue. The isolated embryo, when placed on wet filter paper, did

not germinate but when placed on water agar showed 100% germination. This may have been due to hypoxic conditions occurring when the embryos were imbibing water on the wet filter paper. In Douglas fir the seedcoat seems to assist in water uptake during the early stages of germination (Ching, 1966). Asakawa (1959) found that germination in larch (*Larix kaempferi*) was diminished with an excess of water. Edwards (1969) presented evidence that implicated the seedcoat and the megagametophyte as providing mechanical restraint in noble fir (*Abies procera*) seeds, and Kitzmiller et al. 1975, as well as a number of other researchers (Edwards, 1982) have speculated on some form of seedcoat dormancy in several true fir species.

Chemical inhibitors in the seedcoat may act as barriers against germination (Barnett, 1970; Kelly, et al. 1992). Barnett's group found that loblolly pine seedcoats contained inhibitory activity. However, that activity did not appear to be the major cause of dormancy. Barnett defined his inhibitors as stable chemical compounds that are soluble in methanol, or water, and when concentrated, restrict germination of decoated or nondormant seeds. In the case of loblolly, there seemed to be more inhibitor in the coats than in the seeds themselves. Barnett concluded that other factors, such as seedcoat permeability and mechanical constraint, probably contribute as much or more to dormancy of loblolly pine seeds. If there are inhibitors in the seedcoats of loblolly pine seeds, it may be that these are leached out during chilling treatment and no longer pose a barrier to germination. This hypothesis may have been serendipitously supported by a curious result of the vigorous sterilization procedure

used in this study for excision of the embryo. During this procedure, loblolly seeds were placed in hydrogen peroxide for 1 hour with shaking (see Methods and Materials). When these seeds were placed in the 2±0.5°C chilling treatment it was found that they were germinating precociously. These seeds had a greater percent germination much sooner than did seeds that were sterilized 1 or 2 minutes with hypochlorite bleach. It may be that during the harsh hydrogen peroxide rinse that chemical inhibitors were removed from the seedcoat, thus allowing the embryo to germinate. Another theory is that the seedcoat may have become more porous therefore, allowing oxygen to enter. It was noticed that the color was removed from the hard seedcoat during this procedure. Germination tests in loblolly embryos, following sequential removal of the surrounding tissues, indicated that the hard seedcoat, and not the nucellar cap, the megagametophyte or the inner layer of integument of the seedcoat (fig. 21), played a major role in the physical maintenance of embryo dormancy in the mature, unstratified seed. Only with the removal of the hard seedcoat does complete germination in the embryo occur.

It is plausible to assume that the embryo *in situ* will have many constraints upon it, both physical and biochemical, to prevent germination. The physical constraints mentioned were the tissues surrounding the embryo, especially the hard seedcoat. However, the biochemical constraints have yet to be fully determined. As mentioned previously, chemical inhibitors, hormone-like compounds, and newly synthesized proteins may act to maintain dormancy and/or prevent germination. In this study, the embryo through chilling

treatment did not show the significant shift in protein synthesis that occurred in the megagametophyte. Does this imply that new protein synthesis must occur in the megagametophyte, during dormancy breaking, to allow the *in situ* embryo to germinate? The roles of the newly synthesized proteins of the megagametophyte have yet to be determined.

4.4 Conclusions

4.4.1 The Intact Seed

To summarize this study, it can be stated that loblolly pine seed has a chilling and hydration requirement to break dormancy. The nucleic acids and the lipids remain unchanged during chilling treatment. As well, no significant glyoxylate cycle activity was noted through chilling treatment. The extant protein profiles of embryos and megagametophytes, both buffer-soluble and buffer-insoluble, during chilling treatment showed no changes. The pattern of *de novo* protein synthesis in the embryos showed minor changes through chilling treatment; some proteins were disappearing. However, there was a shift in the pattern of protein synthesis in the megagametophyte extracts through the chilling period that coincided with an increase in germinability. Not only were some proteins disappearing through chilling treatment but many new proteins began to appear. In conclusion it seems that *de novo* protein synthesis is required for breaking dormancy in intact loblolly pine seeds. Future

work will elucidate whether the newly synthesized proteins, possibly in the form of enzymes, remove a germination-constraint or, play the role of enhancers of the germination-gene set.

4.5 Future Research

4.5.1 Nucleic Acids

Further exploration into the causes of dormancy in loblolly pine is required to elucidate at what point regulation of gene expression is occurring. At the nucleic acid level, the study of the message population would help to identify if regulation is transcriptional or translational. It would be enlightening to identify specific mRNAs that are disappearing or appearing as dormancy is lost. Through the use of *in vitro* translation and cDNA synthesis, libraries can be made for screening with probes to follow the patterns of certain messages through chilling treatment. The nature of the probes used to screen, however, has yet to be determined. The results of these findings may act as markers of either dormancy-breaking or germination.

4.5.2 Proteins

Loblolly pine seed showed new proteins appearing as well as proteins disappearing in the megagametophyte at the time of dormancy-breaking. If the appearance of proteins is a prerequisite to germination, then it could be speculated that stopping *de novo* protein

synthesis would inhibit germination. Future work would include experiments with cycloheximide (a potent inhibitor of *de novo* protein synthesis) in the wetting solution through chilling treatment to see if (1) *de novo* protein synthesis is inhibited and (2) if germination will occur without the synthesis of proteins that occurs during chilling treatment. Also, the proteins that are disappearing through chilling treatment should be characterized as to their role in dormancy.

4.5.3 Inhibitors of germination

There are implications in the literature that ABA inhibits germination. Further studies of loblolly pine seed germination should include an experimental system to imbibe isolated embryos in the presence of ABA to see if germination can be inhibited. If it is, then steps can be taken to determine where the regulation is occurring.

Another avenue of interest is the seedcoat itself; does it contain an inhibitor? The vigorous sterilization of loblolly pine seed that resulted in precocious germination of the intact seed would suggest that there is. The other thought is that the seedcoat becomes more porous with the sterilization procedure allowing oxygen to enter the coat. Although increased oxygen content does not necessarily increase respiration it may act to oxidize an inhibitor as suggested by Bewley and Black (1985). This could be controlled for by providing an oxygen-free or a microaerophilic environment to ascertain if germination occurs. An experimental system that looks at components of the seedcoat to determine if one, or a combination of

some, chemicals act as inhibitors could be designed. If an inhibitor is found, it can be used to further explore where and how regulation is occurring.

In this study the tools of molecular biology helped to elucidate biochemical changes occurring during the breaking of dormancy in loblolly pine. Various components of the seed were examined during chilling treatment. Some hypotheses were made about changes that were occurring and some future work was suggested. However, the seed of loblolly is only one stage in a complicated lifecycle. The mechanisms that the tree employs to protect future generations can only be further elucidated by using the tools of molecular biology to study the seed's developmental biology.

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